

STUDY OF ANTI BACTERIAL AND ANTI FUNGAL ACTIVITY OF SOME NOVEL COMPOUNDS



**A
THESIS
SUBMITTED TO THE
UNIVERSITY OF ALLAHABAD
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in
SCIENCE**

**By
UPMA NARAIN**

Under the Supervision of
PROF. (MRS) KRISHNA MISRA

**Nucleic Acids Research Laboratory
Department of Chemistry
UNIVERSITY OF ALLAHABAD
ALLAHABAD-211002**

FEB. 2002

DEDICATED
TO
TEJAS & ARUN

Prof. (Smt) Krishna Mishra
Eminentus Professor



Nucleic Acids Research Laboratory
Department of Chemistry
University of Allahabad
Allahabad-211 002
INDIA
Tel (91)-0532-461236
Fax (91)-0532-607367, 623221
E-mail krishnamisra@hotmail.com
kkmisra@yahoo.com

February 22,2002

CERTIFICATE

This is to certify that the work presented in the thesis entitled "Study of Antibacterial and Antifungal Activity of Some Novel Compounds" has been carried out by Mrs Upma Narain. She has fulfilled the requirement of University of Allahabad,Allahabad,regarding the prescribed period of investigational work for the award of D.Phil degree.

The work included in this thesis is original and genuine.

Krishna Misra
(Krishna Misra)

DR A K BAJAJ

MD, FICAI
Former Professor & Head Skin & VD
MLN Medical College, Allahabad
INDIA

3/6, Panna Lal Road
Allahabad - 211 002

INDIA
Tel. : Clinic: 0532-600561
Res. : 0532-606110
Fax : 91-532-603866
E-mail : bajajak@sancharnet.in

Date : 22nd Feb, 2002

CERTIFICATE

This is to certify that the work presented in this thesis entitled "Antibacterial and antifungal activity of some novel compounds" has been carried out by Upma Narain under my supervision.

The work included in this thesis is original and genuine.



(A.K.Bajaj)

AKNOWLEDGEMENT

I wish to express my foremost and deepest sense of gratitude to my guide and supervisor Professor (Mrs.) Krishna Mishra co-ordinator , Center of Bio- Technology University of Allahabad, (India), for her noble guidance and constant encouragement throughout these research observations under study. . I cannot forget the way she encouraged me in my day to day work for the completion of my thesis . I am equally grateful to Professor A . K. Bajaj , Ex Head Of The Department of Skin & V.D. , M.L.N. Medical College, Allahabad for extending me his able guidance , expert opinion and academic interest as and when required .

I am deeply indebted to Dr. J. K. Gupta, H .O .D . Pathology & Medical Superintendent, Kamala Nehru Memorial Hospital, Allahabad , for inspiring me and permitting me to start and carry on this study without any delay . I am really thankful to him for his kind patronage.

I wish to place on record my heartfelt and sincere thanks to Shri Inder Kumar Khosla, Hony Secretary and Treasurer, K.N.M.H. Society, Teenmurti House, New Delhi for his encouragement and support too.

Undergoing this research work, in the department of chemistry , Allahabad University, Allahabad, I had to consult Dr (Mrs) Geeta Vatal , Sr Lecturer of the department very of and on , I am sincerely grateful to her .I am again equally grateful to Dr (Mrs) Snehlata Tripathi, Mr. Yashvir Singh, Mr. Arvind Mishra, Mr. Sanjay Rai, Miss Vibha Shukla, Mrs Vandana Sonker my research colleagues and other members of Chemistry Department .

All the patients, known and unknown who have formed the basis of this research work and cooperated during specimen collection deserve my heartfelt thanks.

Lastly my special thanks are due to my parents & husband Dr Arun Kant for providing me sincere help and encouragement in the completion of this research .

UPMA NARAIN

CONTENTS

Acknowledgement

Chapter I

Introduction	1-47
1.1 Preliminary concepts	2-5
1.2 Applied importance in therapeutics	5-13
1.3 Basics of chemotherapy	13-28
1.4 Drug action and Drug delivery system	28-36
1.5 Curcumin as a therapeutic agent	36-40
1.6 Objectives	40-41
1.7 References	42-47

Chapter II

Antibacterial activity of some curcumin bioconjugates	48-62
2.1 Introduction	49-50
2.2 Material and Methods	50-52
2.3 Results	52-57
2.4 Discussion	58-60
2.5 Reference	61-62

Chapter III

A case study of 200 patients of Onychomycosis	63-84
3.1 Introduction	64-75
3.2 Material and Method	75-77
3.3 Results	77
3.4 Discussion	77-83
3.5 References	84-85

Chapter IV

Twenty Nail Dystrophy: A case Study of 50 patients	86-94
4.1 Introduction	87-88
4.2 Material and Method	88-89
4.3 Results	89-92
4.4 Discussion	93
4.5 References	94

Chapter V

Antimycotic activity of some curcumin bioconjugates	95-110
5.1 Introduction	96-98
5.2 Material and Methods	99-100
5.3 Results	100-105
5.4 Discussion	106-108
5.5 References	109-110

List of publications

Chapter - 1

Introduction

INTRODUCTION

1.1 PRELIMINARY CONCEPTS

A retreat into the past and going through the ancient medical literature , we realize the importance of natural products . With their wisdom and experience the Alchemists / chemotherapists had evolved a well defined medical system to identify and cure diseases and a treatment schedule which comprised of plant and animal based remedies. With the advent of Allopathy and the introduction of antibiotics, miraculous progress has been achieved in controlling and eradicating contagious diseases. Gradually it led people to leave their native systems of medicine and shift to allopathy and specially antibiotics but these have their side effects too. When we go into the depth of the modern medical system of allopathic remedies we come to know that still a large number of allopathic medicines are made of extracts of natural products including plants simply because such remedies do not leave any side effects or have negligible side effect. Thus the importance of traditional medicine and related writing as sources of remedies, should not be underestimated. We have to look for remedies which give total cure with none or negligible side effects. And to achieve this aim scientists have again approached to those sources which were used for ancient or primitive remedies i.e. natural products.

Primitive writing appeared in the period of Sumerian, Mesopotamian and Egyptian cultures. Historians point out that the men and women of these civilizations (3500 B.C.) had some knowledge about medical techniques even though very primitive. Their curiosity and inquisitiveness helped them to

differentiate plants as non-poisonous or poisonous, that yielded dyes, that were palliative and curative. Though their classification was crude as compared to modern classification, yet it is certain that our ancestors were doing some science, in the literal sense of the word. Ayurveda, one of the ancient system of medical practice, which solely depended on natural products (herbs) can be cited as an outstanding example. This system of treatment was derived from Atharvaveda, where disease and medicines are described as hymns, as early as 2000 B.C. As per the ancient treatise of Ayurveda, Lord Brahma taught Ayurveda to Prajapathi and he in turn, to Ashwinikumars, different sages, Punarvasu, Ateya, Agnivish, Ehela, Jatukarna, Parashar and others wrote text on treatment and medicines of Ayurveda. Charaka's writings, 'Charakasamhita' contains 12,000 verses divided into eight volumes and 120 chapters. Sushrut Samhita, written by Sushrut contains 10,000 verses divided into six volumes and 186 chapters, describing amazing techniques of surgery. Kashyap described in his book, Paediatrics and Obstetrics and Vatsayan wrote on Sexology. Thus a very rich and natural legacy lies dormant in Ayurveda. The essence of Ayurveda is, ***"Everything is medicine. There is no substance in the world which is not medicine. What is important is to know it's exact use"***. The available ancient literature could be summarized as follows. The early man learned to set broken bones, extract and fill in tooth and a variety of specialities like Obstetrics, Ophthalmology, Cardiology, Neurology and even Psychiatry, were in practice. They had found out different bandaging methods to cover wounds of the head, face and battle axe or knife wounds. They used plant and animal products to treat the sick and mortally wounded ones. Of all steps followed, there was a kind of personal relationship developing between

physician and the patient, leading to a positive attitude towards God. The literature also reveals the presence of ethical courts, even discussing physicians fees, after limiting the fees that could be charged either for service on medicine and contained laws providing penalties for malpractice. Widespread use of antimicrobial agents led to the emergence of antibiotic resistant pathogens, which in turn has created an ever increasing need for new drugs. A constant struggle has been going on between pathogenic microorganism and humans since eternity. The pathogens struggle for existence led to change in their metabolism through which they could annul the effect of drugs either by bringing about structural modifications like cleavage of the β -lactum ring of penicillins and cephalosporins or by enhancing their own immunity by alternative means. Thus it is evident that we have inherited resource which are rich and useful in the upkeep of our health and longevity, which are at par with the modern medical practice and ethical courts. What is needed is only to understand the nature and potential of the resources and tap it to suit our present demands.

For this we have to go deep into the most ancient and traditional medical system in India with boldness and generosity and have to find out such sources of remedies which provide us full medical cure with none or negligible side effects. If we succeed in this attempt the act will be a milestone in the interaction between modern techniques and traditional medical systems. Keeping this view in mind the present work is an effort on our part to find out relevant concentrations of curcumin (an active constituent of turmeric) bioconjugates i.e. curcumin linked to an active biomolecule which may have

the potential to act as antibacterial and antimycotic agents. This is the subject of my study reported herein.

1.2 APPLIED IMPORTANCE OF MICROORGANISMS IN THERAPEUTICS

The concept that substances derived from one living organism may kill another (antibiosis) is almost as old as the science of microbiology. Indeed, the application of antibiotic therapy, although started late, however its concept is considerably older. The Chinese were aware, over 2500 years ago, of the therapeutic properties of mouldy curd of soybean applied to carbuncles, boils, and similar infections and used this material as standard treatment in such disorders. The medical literature has for many centuries contained descriptions of beneficial effects from the application to infections of soil and various plants, most of which probably were sources of antibiotic-forming molds and bacteria.

The first investigators to recognize the clinical potentialities of microorganisms as therapeutic agents were Pasteur and Joubert, who recorded their observations and speculations in 1877. They noted that anthrax bacilli grew rapidly when inoculated into sterile urine but failed to multiply and soon died if one of the "common" bacteria of the air was introduced in the urine at the same time. The same type of experiment in animals produced similar results. He commented on the fact that life destroys life among the lower species even more than among higher animals and plants, and came to the astonishing conclusion that anthrax bacilli could be administered to an animal in large numbers, and it would not sicken, provided that "ordinary" bacteria

were given at the same time. *They stated that this observation might hold great promise for therapeutics.*

ANTIBIOTICS

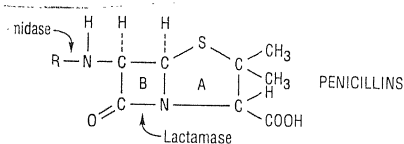
Micro-organism play a pivotal role in producing all groups of antibiotics and have added greatly to our therapeutic armamentarium .The clinical use of antibiotic agents represents the practical, controlled, and directed application of phenomena that occur naturally and continuously in soil, sewage, water, and other natural habitats of microorganisms. During the latter part of the nineteenth century and the early years of the twentieth century, several antimicrobial substances were demonstrated in bacterial cultures and some were even tested clinically but discarded because they proved to be highly toxic.

The number of antibiotics that has been identified are chemical substances produced by various species of microorganisms (bacteria, fungi, actinomycetes etc) that suppress the growth of other microorganisms and may eventually destroy them. Thus they differ markedly in physical, chemical, pharmacological properties, antibacterial spectra, and mechanisms of action. Most have been chemically identified and some have been synthesized. A few are available only as crude or partially purified extracts.

PENICILLINS

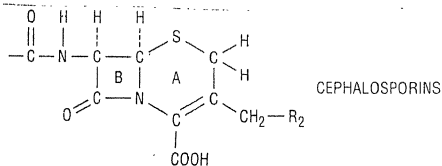
The history of the discovery and the development of penicillin has been recorded by the chief participants(Fleming , 1946 ; Florey , 1946 ,1949 ; Abraham , 1949 ; Chain , 1954). In 1928, while studying staphylococcus variants in the laboratory at St. Mary's Hospital in London, Alexander

Fleming observed that a mold contaminating one of his cultures caused the bacteria in its vicinity to undergo lysis. Broth in which the fungus was grown was markedly inhibitory for many microorganisms. Because the mold belonged to the genus Penicillium, Fleming named the antibacterial substance penicillin



Herrel (1945) records that bedpans were actually used by the Oxford group for growing cultures of P. notatum.¹

CEPHALOSPORINS



Cephalosporium acremonium, the first source of the cephalosporins, was isolated in 1948 by Brotzu from the sea near a sewer outlet off the Sardinian coast. Crude filtrates from cultures of this fungus were found to inhibit the in-vitro growth of *Staphylococcus aureus* and to cure staphylococcal infections and typhoid fever in man. Culture fluids in which the Sardinian fungus was

cultivated were named cephalosporin P, N, and C. With the isolation of the active nucleus of cephalosporin C, 7-aminocephalosporanic acid with the addition of side chains, it became possible to produce semisynthetic compounds with much enhanced antibacterial activity very much greater than that of the parent substance.^{2,3}

BETA- LACTAM ANTIBIOTICS

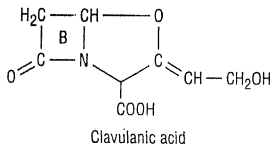
Besides Penicillin there are a few other antibiotics containing beta lactam ring.

Imipenem is derived from a compound produced by *Streptomyces cattleya*.

Azternom is a monocyclic compound isolated from *Chromobacterium violacium*⁴

BETA-LACTAMASE INHIBITOR

Clavulanic acid is produced by *Streptomyces clavuligerus* it has poor intrinsic antimicrobial activity but is a suicide inhibitor of beta lactamase enzyme produced by a wide range of gram positive & gram negative microorganism.⁵⁻⁷

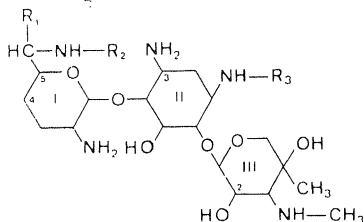


AMINOGLYCOSIDES

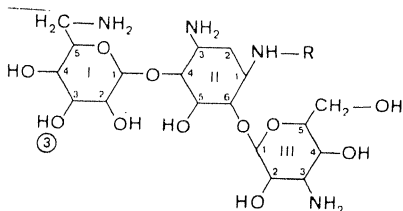
Waksman and coworkers examined a number of soil actinomycetes between 1939 and 1943. In 1943, a strain of *Streptomyces griseus* was isolated that elaborated a potent antimicrobial substance. The first public announcement of the discovery of this new antibiotic streptomycin was made by Schatz, Bugie,

and Waksman early in 1944, and it was soon shown to inhibit the growth of the tubercle bacillus and a number of gram-positive and gram-negative microorganisms in vitro and in vivo.

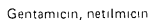
Neomycin- In 1949, Waksman and Lechevalier isolated a soil organism, *Streptomyces fradiae*, which produced a group of antibacterial substances that were labeled "neomycin".



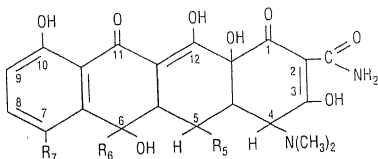
Kanamycin- Kanamycin, an antibiotic produced by *Streptomyces kanamyceticus*, was first produced and isolated by Umezawa and coworkers at the Japanese National Institutes of Health in 1957.



Gentamycin- Is broad spectrum antibiotics derived from species of the actinomycete *Micromonospora*.

NCC1OC(NC2C(N)C(NC3C(N)C(CO)O3)O2)C(O)C1

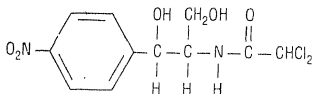
Chlortetracycline and oxytetracycline are elaborated by *Streptomyces aureofaciens* and *Streptomyces rimosus*, respectively.¹⁰⁻¹²



	R_7	R_6	R_5
Chlortetracycline	—Cl	—CH ₃	—H
Oxytetracycline	—H	—CH ₃	—OH
Tetracycline	—H	—CH ₃	—H
Demeclocycline	—Cl	—H	—H
Methacycline	—H	=CH ₂	—OH
Doxycycline	—H	—CH ₃	—OH
Minocycline	—N(CH ₃) ₂	—H	—H

CHLORAMPHENICOL

Chloramphenicol is an antibiotic produced by *Streptomyces venezuelae*, an organism first isolated by Burkholder in 1947 from a soil sample collected in Venezuela.



Chloramphenicol

MACROLIDES

Erythromycin is an orally effective antibiotic, discovered in 1952 by McGuire and coworkers in the metabolic products of a strain of *Streptomyces erythreus*, originally obtained from a soil sample collected in the Philippine Archipelago.¹³

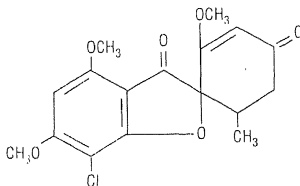
ANTIFUNGAL ANTIBIOTICS:

AMPHOTERICIN B

Streptomyces nodosus, a soil actinomycetes, is the source of two antifungal agents, amphotericins A and B by Vandeputte and coworkers (1956).¹⁴

GRISEOFULVIN

Griseofulvin was first isolated from *Penicillium griseofulvum* diereckx by Oxford and coworkers in 1939¹⁵



Griseofulvin

GENETICALLY ENGINEERED MICROORGANISMS :

Genetically engineered microorganism may have genes incorporated in them that direct the biosynthesis of compounds not associated with normal growth . Techniques for mobilizing genetic information and transferring it between prokaryotic species or prokaryotes & eukaryotes are now established . These techniques induce a microorganism to produce insulin , growth hormone , vaccines and other clinically useful compounds that are useful in treating human ailments .

Human Insulin:

In 1974, research was initiated on incorporating the human insulin gene in *E. coli*. By 1984, commercial production of human insulin by *E. coli* was feasible.

Somatotrophin

Somatotrophin, a human growth hormone used to treat pituitary deficiencies that result in dwarfism. Efforts were made to put the genetic information for synthesis of somatotrophin into *E. coli*.

Vaccines:

One vaccine now obtained from a genetically engineered yeast is that for hepatitis B. The particle HbsAG is not harmful and can be employed as an effective inducer of anti hepatitis B antibody. The protein has been cloned into *Saccharomyces cerevisiae* and is now approved source of the antigen for human immunization.

1.3 BASICS OF CHEMOTHERAPY

Fracastorius of Verona (1546), in his celebrated book on contagious diseases pointed out that the agent of communicable disease was *Contagium vivum*. He put forward the idea that infection itself consists of minute particles and diseases spread by these particles or "seminaria morbid," are too small to be seen by naked eye. He further defined infection and expressed that infectious diseases were transmitted *per contactum* – by direct contact, *per fomitem* – by fomites or inanimate objects and *ad distans* – by air. Von Plenciz (1762) supported the Fracastorian views and explained specificity of living agents in producing various infectious diseases.

The concept of “ spontaneous generation ”or abiogenesis prevailed in India from antiquity and also in Europe since the time of Aristotle to the middle of the eighteen century . According to this theory animals and plants originated from spontaneous combination of elements and ethereal principles .Around mid of the seventeenth century Francesco Redi refuted this theory contending that “ life comes from life .” Redi (1688) conclusively proved it by observing that maggots appeared in decomposing meat where flies laded eggs but when such meat was secured by gauze and protected from flies , there was no maggot formation in the meat .

Aristotle 's theory of spontaneous generation or abiogenesis was supported by Needham , an Irish priest , by extensive experiments . He published his views in a memoir in 1745 and communicated the results of his experiments to the Royal society . He heated meat broth in a container at a temperature sufficient to destroy living organisms and kept the container sealed for some days . On subsequent examinations it was found that the broth swarmed with minute bodies and thus he confirmed the theory of Spontaneous generation of animacules . This was contradicted by Spallanzani (1775) ,an Italian priest , who did not support this view . He prepared flask containing broth and boiled them for varying periods . One set of flasks wassealed and another was corked and these were then stored for several days . On further examination of the flasks it was found that animacules were present in all the flasks except those that were sealed after boiling for sometime . In reply to the absence of any growth in the flasks , Needham stated that the experiments of Spallanzani were fallacious , because some vital substances , termed as “vegetative force” present in the broth , were destroyed by prolonged heating . This criticism ,

however , was finally overcome by Spallanzani by the fact that animacules would grow spontaneously in broth subjected to prolonged high temperature . As a result of further experiments , the doctrine of biogenesis i.e. ‘ life comes from life ’ was conclusively rejected . It was however , Tyndall (1882) who gave the death blow to the theory of spontaneous generation.

Athanasius Kircher (1602-1680), a monk was the first to make direct microscopic study of bacteria in 1658, “minute worms” in the blood of persons suffering from plague. The magnification of the microscope used by him was only thirty-two times and evidently he mistook red blood corpuscles for plague bacilli.

The fundamental observations in bacteriology started from the invention of microscope by Antony van Leeuwenhoek in the last quarter of the seventeenth century and early part of the eighteenth century. He was a Dutch draper and it was his hobby to prepare lenses. He was able to produce an instrument which conceded a magnification of 40 to 300. With the help of this, he used to observe minute organism or “animalcules”, as he called them, in rain water and scraping from the interstices of teeth. Some of these were actively motile. They were round, rod-shaped or bent like sticks or spiral in shape. He communicated the results of his experiments to the Royal Society, London on October 9, 1676. He however did not realize the importance of these animalcules in connection with production of diseases. According to Dobell “Antony van Leeuwenhoek was the first and the greatest of all the microbiologists” who discovered bacteria and spirochaetes in mouth and intestine. 18

Linnaeus (1758) created the genus *Vermes* which he called "Chaos". A few years later, Wrisberg named these organisms *Infusoria*. The *Infusoria* were accurately describe and classified by O.F. Muller, a Danish zoologist, in 1773 and 1786. He introduced the term *Vibrio*. About fifty years later, Ehrenberg (1829) introduced the genus *Bacterium* which meant a "staff". In his book on "Infusion animals", Ehrenberg (1838), described various forms of bacteria, and classified them into *Vibrio*, *Bacterium*, *Spirillum* and *Spirochaeta* but these were considered by him as animalcules. The living nature of yeast globules in beer and wine was determined by Cagniard-Latour, a French Physicist in 1836. Schwann (1837), a botanist, confirmed the living nature of yeasts in beer and wine and found them responsible for fermentation of these liquors. The causative organism of *Favus* was described by Schoenlein in 1837, and Remak cultured the fungus in 1845 and called it *Achorion schoenleini*. In 1857, Nageli noted the relationship between bacteria and fungi and introduced the term *Schizomycetes* for bacteria. The modern classification was introduced by Ferdinand Cohn 1854, when he put bacteria in the plant kingdom. His publications in 1872 marked a great advancement in the systematic study of bacteria. A teacher and Director of research work, he established the foundation of bacteriology on a firm footing.

Further progress of bacteriology was intimately connected with the fermentation industry in Europe and the investigations of Louis Pasterus. In fact, for the development of modern bacteriology and its application, we are indebted to three persons namely, Louis Pasteur, " the father of modern microbiology" Robert Koch, " the father of modern bacteriology" and lord Lister, " the father of antiseptic surgery".

Pasteur was originally a chemist, who undertook the work of faulty fermentation of wine which was seriously affecting the industry of Lille in 1856. It was already established that yeast, a living fungus was the cause of fermentation of sugar to alcohol and Pasteur (1857) fully demonstrated that contaminating rodshaped organisms were responsible for faulty fermentation in which alcohol was being oxidized to an organic acid.

In the course of his researches Louis Pasteur emphasized the importance of scrupulous sterilization of all equipments used for bacteriological investigations. He devised various methods of sterilization namely by the steam sterilizer (16°C), autoclave (12°C), or hot air oven (16°C), by direct flaming and by short exposure to high temperature followed by rapid cooling. The last technique of sterilization is known as Pasteurisation. He has also established the importance of cotton wool plugs for protection of media from aerial contamination, of the composition of nutrient media for bacterial culture, of the reaction of the media and of its oxygen content. After his brilliant work on fermentation, Pasteur (1865-69) was asked to investigate “Pebrine”, a disease of silk industry of France at that time. The question was one of economic importance; here too, microbic (protozoal) aetiology was discovered and practical preventive measure undertaken by him proved effective. This discovery saved the silk industry of France and revived it to a prosperous one.

In 1877 Pasteur worked on the investigation of Anthrax which was rampant and responsible for the death of sheep in France. He prepared anthrax vaccine from attenuated anthrax bacillus (1881) and this proved useful in protection of sheep. He also investigated on chicken cholera (1880) and swine erysipelas

(1882) in relation to recognition of their causative organisms and specific immunization against these infections. He achieved monumental fame by his method of immunization against rabies. The results were very encouraging and in 1888 Pasteur Institute was established in Paris for mass antirabic treatment. These immunological researches established the value of artificial active immunity induced by attenuated living organisms.

Robert Koch, the rival and contemporary of Louis Pasteur, is called "the father of modern bacteriology". Anthrax and its transmission was a puzzling question; because the relation between the organism and the disease was not fully established until the brilliant work of Robert Koch. Pollender (1849) and Davaine (1850) had described minute rod-shaped organisms in the blood of animals suffering from splenic fever. Being inspired by Pasteur, Davaine continued his work and successfully reproduced the disease (1863) in healthy animals by injecting the blood of infected animals. Although Davaine reproduced the disease, it was left for Robert Koch to work it out fully. In 1876 Robert Koch, then a medical practitioner in Woolstein in East Germany, was invited to the laboratory of Cohn, where he experimentally obtained the organism of Anthrax in pure culture in a hollow-ground slide preparation by inoculation of infected blood into the aqueous humour of bullock's eye (1876). He described the spore form of the anthrax bacillus at that time. Koch (1877) prepared dried bacterial film and stained them with aniline dyes for the study of their morphology.

Villemin (1865) had demonstrated that tuberculosis could be transmitted experimentally from man to animal or from one animal to another but Koch (1882) discovered the organism of tuberculosis and in the same paper he

propounded his famous postulates originally mentioned by Henle. Later, the bovine and human types of the organism were differentiated by Theobald Smith (1896). Koch discovered tuberculin in 1890 but it was not universally used for the treatment of tuberculosis. Cholera was a menace and every year a large number of deaths occurred in India, Egypt and other countries as a result of Cholera epidemics. As the head of the cholera Commission, he came to Alexandria, where he discovered *V. cholerae* but as the Egyptian epidemic was declining, he came to India and studied more cases of Cholera in the Medical College, Calcutta. In July 1884, in Berlin Conference, he announced the discovery of the causative organism of Cholera.

The period from 1874 to 1898 marks the golden era of bacterial discoveries, for it was during this period that a rapid succession of phenomenal discoveries took place. These discoveries of organisms of diseases “dispelled the gloom of obscurity of bacterial diseases”. Thus in 1874, Hansen discovered the bacillus of leprosy and in 1879 *N. gonorrhoeae* was discovered by Neisser. In 1881, Klebs described *C. diphtheriae* which was later isolated and described by Klebs and Loeffler and hence the name Klebs-Loeffler’s bacillus. The organism of glanders was described by Loeffler and Schultz in 1882, Gaffky isolated typhoid bacillus which was described by Eberth 4th years earlier. Between 1884 and 1886 Fraenkel and Weichselbaum independently demonstrated the causative organism of lobar pneumonia. In 1886, Escherich isolated the colon bacillus. *Maningococcus* was isolated by Weichselbaum in 1887 and in the same year Bruce isolated the organism of Malta fever. Tetanus bacillus, previously observed by Nicolaier, was isolated by Kitasato in 1889. In the same year Pfeiffer isolated influenza bacillus which he considered to be

responsible for epidemic Influenza and Welch and Nuttall discovered the anaerobic organism *C. Welchii*. In 1894, the organism of plague was isolated by Yersin and Kitasato almost simultaneously. In 1897 Bang discovered the organism of bovine abortion and in 1898 Shiga isolated dysentery bacillus which was named after him.¹⁹

Lord Lister's fundamental studies on bacteria made by Pasteur, were known to Lord Lister, 'the father of antiseptic surgery'. The then Professor of Surgery, in the University of Glasgow, Joseph Lister was deeply interested in the prevention of post-operative sepsis, which took a toll of many lives. He could realize that micro-organisms so prevalent in the atmosphere might be responsible for post-operative inflammation and suppuration. In spite of all sorts of criticism, he followed his own ideas and revolutionized the science of Surgery in 1867 by introducing the antiseptic surgical technique in the post-operative stage. Carbolic acid was chiefly used as spray on the wound or during operation. Results were remarkably gratifying as there was a striking reduction of post-operative sepsis. This was a marvelous achievement of modern surgery and the later development was only a replacement of antiseptic surgery by aseptic surgery which made it possible to avoid the dependence of 'laudable pus' into the security of asepsis.

Bacterial filters were successfully devised by Chamberland (1884) from a cup of unglazed porcelain and subsequently Pasteur-Chamberland filter was utilized for obtaining bacteria-free filtrate containing toxin. Berkefeld filter made of kieselguhr was introduced by Nordmeyer (1891). In 1892 Iwanowski, a botanist, found that an invisible agent, causing mosaic disease of tobacco plants, was present in the filtered juice of diseased leaves. He discovered the

filterable nature of the mosaic disease virus . This was independently confirmed by Beijerinck (1899) , who called the invisible , self – perpetuating agents of mosaic disease as *Contagium fluidum vivum* . Loeffler and Frosch (1898) first demonstrated that the foot and mouth disease of cattle was caused by filterable virus . They found that infective nature of vesicular lymph , collected from foot and mouth disease of cattle , the disease could be successfully transmitted by the bacteriologically sterile but infective filtrate from the lesion . The causal agent which was invisible under the microscope , passed through bacterial filters .

Bacteriophage , in all probability , is the virus of bacteria . Haffkine , in 1896 , discovered self sterilization of water of the river Jumna but that property was lost when the water was boiled . Twort (1915) while working on vaccine lymph first discovered glossy , transparent patches occurring in the white colonies of *Staphylococcus* on an agar plate . He found that filtered material from these colonies could cause transmissible lysis of *Staphylococci* . Such a phenomenon was also observed by D ' Herelle (1917) in dysentery organism and he called this material "*bacteriophagum intestinale* ."By the discovery of electron microscope its particulate nature has been established and it conforms to a bacterial virus in all respects .

Immunological observations were made by the people of ancient India and China in cases of small-pox . Practice of variolation by a process of man to man inoculation was utilized by them to confer immunity against small-pox . This practice was introduced by Lady Montagu in England in about 1721. The method was not a scientific one and in some instances results were disastrous owing to the fact that the inoculated person some times contracted the disease

for which immunity was sought . In 1778 Edward Jener began the study of immunity to small pox . He came to know from the milk –maid who supplied him milk , that they were immune to small-pox ; because due to close contact with cow they were infected with mild doses of cow pox virus which was not virulent as that of small-pox He confirmed this observation and published results of his experiment and in 1798 he put into practice a method of immunization by vaccinia against variola .This gave general acceptance of the principle of active immunization by inoculation of attenuated virus of vaccinia to offer protection against small-pox . About a century later Pastuer(1877) became interested in giving protection by inoculation of attenuated organism and this laid the foundation stone of immunology . Pastuer found that fowls inoculated withold cultures of bacteria of chicken cholera and had mild attacks of the disease , and they subsequently became refractory to infection with a virulent culter . He called these old culters “attenuated virus”.To establish relationship between this discovery and immunity against small-pox by jennerian vaccination , Pastuer introduced the term ‘vaccine’ to various attenuated organisms he used to induce artificial immunity . Pastuer then took up the work of anthrax vaccine which was prepared by attenuating the organism at 42 to 43⁰C. ; this also proved to be successful against anthrax in sheep and cattle . Pasture realized that the infection of rabies was caused by an invisible virus . He attenuated the virus at first by passing the organism through rabbits and then by drying the infected spinal cord of the animal . He prepared vaccine from the attenuated virus and this Pasture antirabic vaccine has since been used for the prevention of hydrophobia . In 1881 Metchnikoff , a Russian zoologist , observed the defensive mechanism of Daphnia , a water

flea , in its transparent body . He observed that in its struggle with an invading fungus , mobile cells of the body played the role of engulfing and digesting the organism . This observation laid the foundation of cellular theory of immunity was based upon Nutall's convincing demonstration of bacterial property of blood in 1888 . Thus the schools of immunology were established and both these schools made rapid progress on the subject with extensive experimental data . By the end of the last century , many immunological reactions were discovered , namely agglutination , precipitation , complement fixation , etc . In 1896 – 1899 Ehrlich introduced his famous side – chain or receptor theory of antigen – antibody reactions and this dominated the thoughts on immunity for about twenty five years . Ehrlich wanted to explain antigen – antibody reactions on the basis of chemical reactions , whereas Bordet concluded that serological reactions could be explained by the absorption theory .

Another important discovery in immunity was the discovery of specific hypersensitiveness . In 1837 , Magendie , a physiologist , described sudden death of dogs after repeated injections of egg albumen . Flexner (1894) made an assimilar observation . He observed that the animal which would withstand the first injection of egg albumen , would succumb to a second injection of the egg albumen administered after the lapse of some days ; but the systematic study of hypersensitiveness began in 1902 when Richet and Pointer carried out the first systematic study of anaphylaxis . Arthus phenomenon was published in 1903 and Theobald Smith phenomenon was communicated to Ehrlich in 1904 . the latter is a state of sensitiveness of the guinea pig after second and third injection of diphtheria toxin- antitoxin when the inoculations were widely spaced . This phenomenon was later explained by Otto (1905) owing to the

presence of horse serum in the mixture which produced sensitization .The study of the manifestation of hypersensitiveness started from Jenner's observation of accelerated vaccinia reaction which he observed in the skin of certain vaccinated subjects In 1905 von Pirquet and Schick published their observations on serum sickness . All these focused the attention of investigators on the problem of hypersensitiveness but the question is still a baffling one and not yet fully explained in all its aspects .

Roux and Yersin (1888) demonstrated toxin in the filtrate of the broth culture of the diphtheria bacillus and attributed the symptoms of diphtheria to the action of soluble toxin produced by the bacillus . Knud Faver (1890) demonstrated that tetanus bacillus also produced a toxin similar to one produced by diphtheria bacillus. In 1890 , Emil von Behring observed that the animals could be protected against diphtheria by injection of diphtheria toxins in small doses . Therefore it is Behring who discovered the method of immunization with toxin producing antitoxin against diphtheria . Behring and Kitasto (1890) discovered antitoxin for tetanus . Roux and Martin (1894) subsequently immunized horses to prepare antitoxin in large scale . First serum treatment in man was introduced by Behring and Wernicke and the first dose of antitoxin was administered to a human subject on the 25th December , 1891 . This marks the red letter day in the history of specific therapy . This life saving measure is still the sheet anchor for the treatment of diphtheria which till the first world war took a heavy toll of children .

In 1889 Charrin and Roger observed that *Ps. Aeruginosa* , a motile organism , when it came in contact with the immune rabbit serum , first lost the motility and then formed the agglutinating clumps. This was confirmed by other

workers in different organisms . This knowledge was utilized by Widal and his collaborators (1896) who applied the reaction in the diagnosis of typhoid fever to detect antibodies in patients and the test is now known as Widal test . The agglutination reaction also helps in the identification of unknown organisms or types of organisms to show their antigenic differences . Bordet (1898) utilized this reaction to specific reaction of red blood corpuscles with anti serum . Landsteiner (1900) utilized agglutination reaction in the determination of blood groups , the knowledge of which is so perfect today that safe transfusion of blood has now become a routine practice in hospitals .

Pfeiffer (1894) observed that when *V. cholerae* was introduced in the peritoneal cavity of a guinea pig which had recovered from inoculation of *V. cholerae* , the organism is promptly agglutinated and lysed . The same phenomenon was observed when the *Vibrio* was mixed with immune serum and injected into the peritoneal cavity of a guinea pig . This lysis of bacteria is known as “ Pfeiffer’s phenomenon”. A few years later , Bordet (1898-1899) observed lysis of red cells in the presence of an anti red cell serum and established the relation of complement to lysis of cells . It is now known that in the absence of complement only agglutination or sensitization occurs without any lysis . He demonstrated that in specific antigen antibody reaction in presence of complement , the latter was absorbed by this antigen-antibody complex . Bordet and Gengou (1901) and Gengou (1902) used this immuno-haemolytic system as a delicate means of detecting the specific antibody in the presence of the known antigen . They showed that when an antigen –antibody reaction occurred , complement was fixed by the antigen – antibody complex and the sensitized red cells of a haemolytic system suffered haemolysis . Thus

the haemolytic system was utilized as the most delicate indicator for the detection of antigen-antibody reaction. The Bordet- Gengou phenomenon was utilized by Wassermann, Neisser and Bruck (1906) for the serodiagnosis of Syphilis and the test is nowadays known as Wassermann reaction. Rudolph Kraus (1897) observed that when the filtrate of cholera culture was mixed with cholera antiserum, a precipitate formed rapidly at the interface and soon floccules appeared which subsequently settled at the bottom of the tube. Such reactions were also observed with the filtrate of typhoid and plague cultures. This reaction, known as precipitation, was highly and promised a very important implication in the mechanism of immunity reaction.

Chemotherapy, as an antibacterial measure, developed chiefly in three stages. The pioneer worker in this field was Paul Ehrlich. Ehrlich observed that organic arsenicals killed Trypanosomes in an infected animal but if smaller non-effective doses were administered, the trypanosomes rapidly acquired tolerance to the drug. Therefore he aimed at "Therapia magna sterilans" i.e. the introduction into the blood of a single dose of chemotherapeutic agent, sufficient to kill the parasite. He however, did not succeed in this respect, but on the other hand he found that the drug undergo certain changes in the body after which it would produce the desired action. Ehrlich and Hata (1910) discovered "Salvarsan" and administered it successfully in cases of syphilis.

The next stage was the introduction of prontosil by Domagk. This opened a new avenue towards the development of chemotherapy in the realm of bacteriology. Domagk reported the effect of prontosil rubrum, an azo dye, against streptococcal infection and this was confirmed by various workers in France and England. In 1936 it was found that prontosil was converted into

sulphonamide in the body which was the active agent responsible for its action against streptococci. This was, however, followed by the discovery of safer and more effective sulphonamides. Sulphones have since been introduced and are eddective against acid fast bacilli.

The latest in the field of chemotherapy are the antibiotics. The knowledge of ingibiting action of one organism against another (antibiosis or antagonism) has been utilized in the treatment of infections. The substance isolated from one organism and used to inhibit the growth of another organism, is known as antibiotic. The action of pyocyanin derived from *Ps. Aeruginosa* was known before, but no head-way progress was made in the direction of utilizing these bacterial products against microorganisms. Fleming (1929) observed that *Penicillium notatum*, a laboratory contaminant, growing on agar plate, inhibited the growth of staphylococcus growing in the same plate. An extract of this substance was used by him locally for dressing of wounds in 1932. This discovery was almost but isolation of gramicidin in 1939 by Dubos and isolation from *Bacillus brevis* created a new interest in the subject and during the World War II., the Oxford group of workers, namely Florey, chain and their associates revived the work of *P. notatum* and isolated penicillin from this fungus. Since then many antibiotics have been worked out and the most important of these are streptomycin, discovered by Waksman from *Streptomyces griseus*, bacitracin from *B. subtilis* by Melency and other antibiotics like chloromycetin, aureomycin, terramycin, etc. These are now in extensive use in clinical practice and have almost replaced sera, vaccines and other chemotherapeutic drugs.

Microbiology owed much of its early recognition to its usefulness in medicine, agriculture and industry. But it soon became obvious that the bacterial cell and its products had many attractions for the general biologist, the chemist, the physicist, the geneticist, the pharmacologist and other scientists as well as the bacteriologist, immunologist and pathologist. In its free living form, the bacterial cell with its simple food requirements, rapid growth and remarkable plasticity, and its great range of enzymes and diffusible products is very suitable for detailed study and, in the past few decades, bacterial physiology (sometimes called microbial chemistry) has made many contributions to advances in our knowledge of cellular and molecular biology. Together with bacteriophage (its own particular parasite) the bacterial cell has been specially useful in advancing the science of genetics while the biochemist interested in enzymology finds it a most valuable granary.

The last three decades have not only witnessed the rise and development of those most brilliant chapters of medicine, infection and immunity; but sanitation, agriculture, many industries and other fields of human activity have benefited largely by the development of bacteriology. The most important problem for the future is to create a system of Bacterial Therapeutics of equal efficiency.^{20,21}

1.4 DRUG ACTION AND DRUG DELIVERY SYSTEM:

The active ingredient in a medicine is only part of the arsenal against disease, the drug must somehow get to the **“right place at right time in right concentration”**. That’s where drug delivery comes in. Historically the treatment of disorder was empirical and the only way of knowing whether or not a drug would work was to try it. By process of trial and error, the best drug

and the best dose was defined for the patient.²² The future hope and promise of pharmacogenetics is that by understanding the molecular basis of individual variation in drug response, knowledge will be gained on how to focus on the patient as an individual, defining the medicine/drug and dose most suited to a patient before prescription. In reality it is known that environmental factor will also play a significant role in defining drug response²³, molecular basis of complex phenotype of drug response will be long and complicated process. According to receptor site theory the locus on the cell membrane has same geometrical dimension as that of drug molecule and has the capability of holding the drug molecule on its surface by any type of forces. Thus, the action of drug on a cell depends on its molecular structure and is not affected by physical properties like solubility, etc. The special configuration of drug molecule and specially the nature of substituent group present play an important role. The molecule of a drug must fit into protein template on the plasma membrane like lock and key. Therefore, a slight variation in the fundamental structure of the molecule is sometimes sufficient to annule the activity of the drug²⁴. It is important to recognize that drug response is a complex phenotype to which all probability series of genetic and environmental factors also contribute. To date the relative ratio of these components and importance of their interaction is not known. There is likely to be significant truth in the statement that if we are to define the genetics of drug response we need first to define the gene for compliance. Suffice to say, there is currently a great deal of debate regarding the feasibility of identifying genetic association with complex phenotype, but much effort is being invested to identify tools that will improve the chance of success^{25,26}. The chance of

success also depends on the toxicity of drug i.e., the better drug is one which delivers at right site with right toxicity i.e. toxic enough to cure disease but not too toxic to cause undesirable side effects on healthy cells and tissues. The optimum toxicity can be achieved by suitable chemical modification of the drug molecule. However, its delivery at the required site mainly depends on achieving the correct affinity by either proper chemical modification or by attaching it with a suitable ligand.

TARGETED DRUG DELIVERY

The value that drug delivery adds can be improved safety, efficacy, convenience and patient compliance. When a drug is administered in body by any means, it diffuses throughout the body but there are certain specific sites where the drug is required in an appreciable amount to be effective. If by any means the drug can be administered or enriched at the site where it is required (target) is known as targeted drug delivery. For such type of process the foremost important thing is selection of target e.g. in case of cancer, almost all cancerous cells are hypoxic in nature i.e. the normal cells have normal level of oxygen whereas cancer cells have extremely lower level of oxygen²⁷. So, the drug can be designed in such a way which can be toxic only in cells containing lower level of oxygen. In the present work described in this thesis we have targeted hypoxia i.e. designed the bioconjugates in such a manner that drug can be converted to a free radical drug intermediate (toxic drug). Which has sufficient time inside the cells containing lower level of oxygen and due to the toxicity the cell death occurs. Whereas inside the cells containing normal level of oxygen the free radical drug intermediate gets converted gets

converted to parent molecule (non-toxic). Hence in cancer patient the hypoxia can be the target in our present work we have tried to achieve this goal.

According to central hypothesis the target can be identified by the use of genetics, an understanding of the underlying molecular defects in common clinical disorders will be achieved and therefore novel improved therapeutics will be discovered that will target these defects. The term target characterization refers to the use of genetic analysis to define the variation within a gene encoding a molecule identified as a potential drug target e.g. telomer sequences. The Telomeres are specialized heterochromatin structure that act as caps at the end of chromosomes. In human, telomeres are made up of an average 5000-15000 base pairs of [TTAGGG] $_n$ repeats^{28,29} and telomeres binding proteins. The exact number and combination of base pairs in these telomer repeat sequences varies slightly between species but their function is same:

1. They form specific complexes with telomer binding protein.
2. They protect chromosome end from exonuclease digestion.
3. They prevent aberrant recombination.
4. Telomeres prevent chromosomal end from activating checkpoint control that sense DNA damage³⁰.

Because of inherent flaw in the way the cells copy their DNA, each time a cell divides it loses 50-100 base pairs at the end of its telomere. This is known as end replication problem, as first described by Watson, and is consequence of the polarity of DNA strands and the mechanism of DNA synthesis³¹. When

telomere losses a critical number of base pairs, it triggers a signal for the cell to stop dividing and senescence³². Cells have evolved a number of strategies to counteract this progressive telomere loss, including complex recombination and retrotransposition schemes. But most common solution in higher eucaryotes is an enzyme complex called as Telomerase³³, a specialized reverse transcriptase that specially adds back telomere sequences that are lost during replication. This process maintains a dynamic equilibrium and prevents the chromosomes from receiving the signal to stop dividing. The cells that produce telomerase includes germ cell and cancer cells, the normal somatic cell lacks telomerase activity²⁸ and hence can be targeted.

Drug Delivery Systems

Some latest drug delivery systems have made treatment more focused. With several new techniques, the relatively higher concentration of drug can be delivered to a specific diseased tissue, which allows greater potency and less toxicity to health tissue. The most novel drug delivery systems have stemmed from work on new polymers, lipid vesicles, cyclodextrins, prodrugs and viral vectors.

Polymers

The drug when linked with polymers slowly into the blood stream over a long period. Levonorgestrel implant hormones are of polymers that produce sustained drug level. Polymer linked drugs that are deliverately oversized can

also better target diseased tissue by escaping through leaky micro vasculature and then entering cells by endocytosis. In a model of melanoma, this approach has enabled researchers to achieve levels of the anti neoplastic drug doxorubicin that are upto 70 times higher in tumor tissue than those achieved with conventional formulation. Researchers are also perfection drug polymer complexes that are linked to antibodies for “active tissue targeting”. Thus a drug polymercomplex can be designed to undergo a conformation change or enzymatic breakdown that result in release of active drugs only under certain conditions. Similar advances in chemistry have allowed many drugs to be administered through the skin i.e. transdermal delivery. The drug delivery through the skin has the advantage of maintaining consistent blood level. Patches that can be worn for several days also appears to help compliance. The hormones that are effective at low concentration are good candidates for transdermal delivery systems. But patches are not ideal for delivery of large doses of drugs that are needed in antibiotic therapy and also not economical for such low cost drugs like aspirin.

Liposomes

The liposomes are another construct that serve as an envelope for active drug particles and can, like polymer, deliver high concentration of drug to infected or neoplastic tissue. Liposome delivery systems are similar to polymer system but the drug is encompassed by a vesicle instead of being physically linked to a polymer. In liposome a hydrophilic drug can be trapped in aqueous channels that course between successive phospholipid bilayers, whereas hydrophobic

medicine can reside within the bilayer itself. The nontoxic, nonimmunogenic bilayers dissipate, allowing some diffusion of active medicine a method widely used. The liposomal formulation for daunorubicin and doxorubicin were recently approved by the food and drug administration for treatment of HIV- associated Kaposi Sarcoma.

Cyclodextrins

Another chemical innovation currently being used for drug delivery involves the taming of naturally occurring class of molecules known as cyclodextrins, which were once too toxic to be used in medicines. A cyclodextrin is composed of dextrose units held together by a lipophilic core and hydrophilic exterior. Orally cyclodextrin are fine, but with injection they are nephrotoxic, which can be engineered as a modification of cyclodextrin to decrease their toxicity. The cyclodextrins are used in the formulation of antipsychotic drugs for example, in a new formulation of intracranazole.

Prodrugs

Prodrugs³⁴ are therapeutically inert when administered but become bio-active at or near the site of action inside the body. The prodrugs are easy to deliver at the site of action and are prepared by linking an active drug with an active site to a ligand by a suitable biodegradable bond like ester, amide, anhydride etc. These biodegradable bonds get degraded inside the body in presence of suitable enzymes and soon the inert drugs gets activated. Fosphenytoin is to be

sold as cerebyx, a reformulation of the anti-convulsant, phenytoin that can be given safely by intravenous injection. Conventional phenytoin can cause cardiovascular collapse when given parenterally. The Pro-drug concept is popular: About 15% to 20% of new approved drug are Prodrugs.

The oligonucleotide Prodrugs have also been designed and synthesized, which would undergo enzyme mediated transformation within target organ to release the oligonucleotide³⁴. The prodrug design in this instance involves the conversion of hydrophilic oligonucleotide into a lipophilic one and less ionic oligonucleotide conjugate, which would enter cells by passive diffusion through cell membrane and also be transported across various physiological barriers, including the blood-brain barrier. It has been proved that suitable bioreversible analogue of phosphorothioate oligonucleotides, which carry one or more of the lipophilic carboxylic ester group i.e. S-alkyl phosphorothiolates be a novel prodrug forms of the parent phosphorothioate³⁵.

Gene Delivery

Physician-scientists have devoted attention to gene replacement in monogenic deficiency diseases, such as cystic fibrosis and to many strategies for manipulation the genomes of cancer cells. Getting the right DNA into right tissue is challenge. The vector gene delivery system can be broken into two areas: viral and nonviral. Viral is the dominant use because viruses are the most efficient parasites according to Nelson Wivel, Deputy Director of the Institute for Human Gene therapy at the University of Pennsylvania. Wivel said that early work using disabled retroviruses failed because of inability of

the retrovirus to infect non-replicating cells. Adenovirus vectors are now more common although they are more vulnerable to host immune systems and their longevity of transcription is less. Non viral vectors for gene therapy, such as liposomes that are combined with plasmids or DNA have been studied with very low efficiency of expression, many of the liposomes are absorbed by the cells and degraded. The tissue specific targeting is the Holy Grail of this field.

The rationale behind the systemic drug delivery is to achieve greater efficacy with lower toxicity. Enhancing the specificity of therapeutic drugs and thereby improving their site delivery is the primary goal of today's pharmaceutical industries. The Past decades have seen extensive use of liposomes and lipid based delivery systems to improve the pharmacological properties of a variety of drugs³⁶⁻⁴².

Lipid-oligonucleotide complexes can provide level of encapsulation but their size and charge severely impair their ability to distribute systemically after parental administration and subsequently extravasate to disease site. As a result this type of liposomal carriers have a limited utility for the treatment of systemic diseases⁴³. Also the intravenous delivery of lipid – DNA complexes can also induce strong toxicity and results in considerable mortality⁴⁴⁻⁴⁶.

1.5 CURCUMIN AS A THERAPEUTIC AGENT

The word bioconjugation is defined as the linking of any biomolecule with therapeutically active molecules. Keeping this rationale in mind ,bio conjugates comprising of nucleosides and amino acids with a suitable molecule which is itself biologically active against inflammatory disorders and

devoid of any charge were synthesized in our laboratory. Nucleosides and amino acids rapidly cross the plasma membrane of the cells by a facilitated transport mechanism⁴⁷, thus gaining rapid entry into the cells. To get the rapid entry into the cells curcumin has been derivatised/modified to its bioconjugate form. Multiple therapeutic efficiency of curcumin facilitates the development of its bioconjugates, might change the way in which curcumin enters the cancer cells. The relatively small bioconjugates may usually enter the cancer cell by a process of diffusion i.e. they gradually seep through the cell membrane. While such delivery process may have some effectiveness the first time cancer cells are attacked through chemotherapy, on subsequent treatment the cells often develop a resistance not only to the particular drug, but other therapeutic agents as well. This multidrug resistance has long been a problem in the treatment of cancer⁴⁸.

Curcumin molecule, meeting such requirement was chosen for dual purpose. It is isolated from turmeric, the powdered rhizome of plant *Curcuma longa* and has been extensively used for centuries as colouring agent, spice and as traditional medicine for external/internal wounds, liver diseases, (preferably jaundice), blood purification, in treatment of inflammatory conditions and other diseases⁴⁹⁻⁵². Curcumin has also been reported to have anti-oxidant properties⁵³⁻⁵⁴. Turmeric and curcumin have been shown to inhibit carcinogen induced mutation in the Ames assay and the formation of tumor is several experimental systems, suggesting anti-initiating and / or anti-promoting activity against several chemical carcinogens⁵⁵⁻⁶². In addition turmeric and curcumin have also have been shown to significantly inhibit B(a)P (Benzo [a] pyrene) –induced forestomach tumors^{56,58,61} and DMBA (7,12-dimethylbenz

(a) anthracene) induced mammary tumor in rat^{59,61}. Curcumin free aqueous turmeric extract (CF ATE) have also been shown to inhibit B(a) P-induced forestomach papillomas in mice^{66,65}. Recently it has been reported that curcumin decreases, total cholesterol and LDL cholesterol level in serum and also increases the beneficial HDL cholesterol level. Curcumin is reported to inhibit the proliferation of HUVEC (Human Umbelical Vein Endothelial Cell). Therefore it could turn out to be a useful compound for the development of novel anti-cancer therapy⁶⁶. It has also been claimed that curcumin inhibits HIV-1 integrase protein, thus potentially preventing HIV-1 from infecting CD-4 & CD-8 cells⁶⁷. The attractive features of curcumin to explore as vulnerary agent is that despite of being eaten daily for centuries in Asian countries, curcumin has not been reported to be toxic⁵⁰

Curcumin molecule has interesting structure with two phenolic and one active methylene function, which are the potential sites for attaching bio-molecules. Since the phenolic group is responsible for the antioxidant activity of curcumin, the covalent linking of any biomolecule with curcumin through phenolic group may retard the antioxidant activity. Keeping this point in mind we have linked biomolecules through phenolic group of curcumin via a biodegradable bond like ester or amide. These bonds are highly biologically sensitive and once inside the body these may be degraded in presence of suitable enzymes resulting in releasing the curcumin molecule to the target organ.

The rationale behind the synthesis of bioconjugates of curcumin was that curcumin is a therapeutically active molecule as has been discussed earlier, but its poor absorption through the intestinal wall requires repeated dosage &

limits its wide application. As we know from the ancient time people use curcumin (turmeric) for external/internal wounds and other variety of diseases by mixing curcumin (turmeric) in sugar, honey and milk. This concept was probably to enhance its poor absorption, so that maximum amount can be utilized for therapy. So, we have opted one of the accessible approach to enhance its delivery without affecting the activity of this molecule i.e. make its bioconjugates by the covalent attachment with such ligands which can easily internalize with cellular environment of the pathogen. Since amino acids (glycine & alanine) and nucleosides (uridine/thymidine) are essential components of bacterial cell and when linked covalently to curcumin may act as carrier molecules and thereby increasing intracellular delivery of curcumin. Henceforth, the amino acid curcumin bioconjugates & nucleosides curcumin bioconjugates were synthesized⁴⁸. These bio conjugates serve dual purpose i.e. of systemic delivery⁴⁸ as well as acting as therapeutic agents . The size of curcumin bioconjugates as compared to liposomes(>1-2 μm) is small enough to get through the passage in pulmonary capillaries and the approach for improving the uptake involve such a design which would undergo, enzyme mediated transformation within the target organ. The conjugate bond reported herein in case of amino acid curcumin bioconjugates are exclusively enzyme sensitive i.e. Prodrug to cause a positive delivery.

Numerous reports have shown that as cells enter the S-phase, the activity of many enzymes involved in DNA synthesis increase, including thymidylate synthetase (T.S.) and thymidine kinase (T.K.)⁴⁹. Reddy et. al. have also shown that both T.S. and T.K. were significantly inhibited by the initiator of DNA synthesis⁷⁰. However, there was a slight inhibition of T.S. activity with

significant loss of T.K. activity upon curcumin treatment that correlates with the significant inhibition of DNA synthesis⁶⁶. Formation of new blood vessels is a highly controlled process which involves the endothelial cell migration, proliferation and production of enzymes capable of modifying the extracellular matrix under the physiological conditions, new blood vessels are formed during the body's repair process⁴⁴ such as wound healing and embryonic development⁶⁷⁻⁷¹. However neovascularisation is widely associated with a variety of pathologies, including tumor growth⁷², atherosclerosis⁷³ rheumatoid arthritis and inflammation⁷⁴. The inhibition of proliferation and angiogenic differentiation of HUVEC on matrigel by curcumin has shown many promises for treatment of angiogenic diseases⁶⁶. Gold salt and D-Penicillamine inhibit neovascularisation in vitro and in vivo.⁷⁵⁻⁷⁶ Recently methotrixate, which is a potent folate antagonist, was found to inhibit in vitro endothelial cell proliferation and in vivo neovascularisation⁷⁷⁻⁷⁸. The unique action of curcumin on T.K. cell cycle progression and further understanding of mechanism of action on endothelial cells could ultimately lead to development of curcumin and its bioconjugates as anti angiogenic drugs.

1.6 Objectives:

The overall objectives of the present work are as follows:

1. To prepare the serial dilution of curcumin & curcumin bioconjugates using organic solvents and water.
2. To screen the prepared dilutions for in vitro antibacterial property using following assays:

i.Determination of Minimum Inhibitory Concentration
(MIC) by Macro dilution method.

- ii. Calculation of Zone of Inhibition (ZI) by Disk Diffusion Method.
 - iii. Correlation of MIC's with ZI.
- 3. To screen the prepared dilution for anti fungal property against fluconazole resistant onychomycosis agents.
 - i. Determination of Minimum Inhibitory Concentration (MIC) by Macro dilution method.
 - ii. Calculation of Zone of Inhibition (ZI) by Disk Diffusion Method.
 - iii. Correlation of MIC's with ZI.
- 4. A case study of 200 patients of onychomycosis .
- 5. 20 nail dystrophy : A case study of 50 patients.

REFERENCES

1. A. Braham, E.P. The action of antibiotics on bacteria In, Antibiotics, Vol II Oxford University Press, New York, 1949, P 1438-1496.
2. The cephalosporins. Pharmacol. Rev, 1962, 14,473-500.
3. Tarly, F.P.; Jacolus, N.V.j, In vitro activity of N-formidoyl thienamycin (MK0787) Antimicrob. Agents Chemother., 1980,18,642-644.
4. 3 Skyes, R.B., and others. Monocyclic B-Lactam antibiotics produced by bacteria, Nature, 1981, 291, 489-491.
5. Neu, H.C., and Fu, K.P. clavulanic acid, a novel inhibitor of B-lactamases Antimicro. Agents chemother, 1978, 14,650-655.
6. In vitro and in vivo synergism between amoxicillin and clavulanic acid against ampicillin resistant Haemophilus influenzae type b. Antimicro. Agents Chemother, 1981, 19, 993-996.
7. Bull, A.P.j Geddis, A.M.; clavulanic acid and Amoxycillin: a clinical, Bacteriological and pharmacological study. Lancet, 1980, 1,620-623.
8. Waksman, S.A. (e.d.). streptomycin. Nature and Practical Application. The Williams & Wilkins Co., Baltimore, 1949. Rosselot, J.P.j Masquez, E.j Murawski, A.; Hamdani A. et al. Isolation, Purification and characterization of gentamicin. In Antimicrobial Agents and chemotherapy – 1963. (Sylvester, J.C. ed.) American Society for Microbiology. Ann Arbor, Mich., 1964 PP 14-16
9. Higgins, C.E., and Kastner, R.E. Nebramycin. a new broad spectrum antibiotic complex. II Description of Streptomyces tenebrarius. Antimicrob. Agents Chemother., 1967, 7, 324-331.

10. Dowling, H.F. Tetracycline. Medical Encyclopedia. Inc., New York, 1955
11. Lepper, M.H. Aureomycin (Chlortetracycline). Medical Encyclopedia. Inc., New York 1956.
12. Bartz, Q.R. Isolation & characterization of Chloromycetin J. Biol. Chem., 948, 172, 445-450.
13. Steigbigel, N.H. Erythromycin, Lincomycin and clindamycin. In. Principles and practice of Infections disease, 2nd ed-(Maxdell, G-L; Douglas, R.G., Inc. New York, 1985, PP – 224-232.
14. Vandeputte, J.; wachtel, J.L.; Amphoterics A and B. antifungal antibiotics produced by a streptomyces. II . The isolation and properties of the crystalline amphotericine. In. Antibiotics Annual. 1955-1956. Medical Encyclopedia . Inc. New York. 1956 PP 587-591.
15. Symposium [Various Authors] Griseofulvin and dermato mycoses Arch Dermatol, 1960,81; 650-882.
17. Collected Memories of Anton V. Leeuwen Hock, Royal Society of London, 1675, 1683.
18. Brock, T.D. (1961) Mile stones in Microbiology Translated by N.J. Englewood. London: Prentice Hall.
19. Bolloch, W. (1960) The History of Bacteriology. London: Oxford University Press.
20. Dubos, R. (1950) Lows Pasteur, Free lance of Science Boston: Little Brown.
21. Godlee, R.J. (1917) Lord Lister. London. Macmillan.

22. Patsalos, P.N. *Ther. Drug. Monit* 22, 2000, 127-30.
23. Poolsup, N., *J. Clin. Pharm. Ther.* 25, 2000, 197-220.
24. Misra, K. and Dubey, R.C. In *Chemistry of Synthetic Drugs*, South Asian Publishers, New Delhi, 1994-1-23.
25. Kruglyuak, L., *Nat. Genet.* 22, 1999, 139-144.
26. Schork, N.J., *Am. J. Respir. Crit. Care, Med.* 156, 1997, 5103-5109.
27. Brown, J. M. *Molecular Medicine Today*. 6, 2000, 157-162.
28. Katherine E. McKenzie, Christopher B.U., Saraswati S., *Mol. Med. Today*, 5(3), 1999, 114-122.
29. Hayflick, L. *Exp. Cell. Res.* 37, 1965, 614-636.
30. Blackburn E.h., *Biochemistry (Mosc)*. 62 1997, 1196-1201.
31. Watson J.D. *Nat Biol.* 239, 1972, 197-201.
32. Harley, C.B. Futcher, A.B. and Greider, L.W., *Nature*, 345, 1990, 458-460.
33. Nugent, C.I., and Lundblad, V., *Genes Dev.*, 12, 1998, 1073-1085.
34. Bundgaard, H., In *Bioreversible carriers in drug design. Theory and application* (Roche, E.B., Ed.) Pergamon Press, New York, 1987, 13-94.
35. Radhakrishnan, P.I., Dong, Y., and Agrawal, S., *Biorganic Chemistry*, 23, 1995, 1-21.
36. Perlaky, L., Saijo, Y., Busch, R.K., Bennett, C.F. Mirabelli, C.K., Crooke, S.T. and Busch, H. *Anti-cancer Drug Design*, 8, 1993, 3-14.
37. Saijo, Y., Perlaky, L., Wang, H., and Busch, H. *Onclo. Res.* 6, 1994, 243-249.

38. Marzo, A.L., Fitzpatrick, D.R., Robinson, B.W.S., and Scott, B.,
Cancer Res. 57,1997,3200-3207.
39. Sacco, M.G. Barbieri, O., Piccini, D., Noviello, M., Zucchi, I., Frattini,
A., Villa, A., and Vezzoni, P., Gen. Ther., 5,1998,388-393.
40. Gokhale, P.C., Soldatenkov, V., Wang, F.H., Rahmen, D., Drischilo,
A., and Kasid, K., Gen. Ther. 4, 1997,1289-1299.
41. Litzinger, D.C., Brown, Wala, I., Kaufman, S.A., G.Y., Farrel., C.L.,
and Collin, D. Biochim., Acta., 1281,1996,139-149.
42. Hope, M.J., Bally, M/B., Webb, G., and Cullis, P.R. Priochim.
Biophys. Acta., 812,1985,55-56.
43. Litzinger, D.C., J Liposome Res. 7, 1997,51-61.
44. Huang, L., and Li, S. Nat. Biotechnol., 15, 1997, 620-621.
45. Li, S., Am. J. Physiol, 276,1999, L796-L804.
46. Dow, S.W., J. Immunol, 163,1999,1552-1561.
47. Robins, R.K., and Revanvar, G.R., Design of Nucleoside Analogues as
Potential Anti viral agent, P. 11-36, Antiviral Drug Development: A
multidisciplinary Approach (Ed: Erik De Clercq ad R. Walker), 1988,
Plenum Press, New York.
48. <http://www.pharmaceutics.utah.edu/kopecek/news/new.htm>
49. Kumar, S., Narain, U., Tripathi, s., and Misra, K. Bioconjugate
Chemistry, 2001.(In press).
50. Govindrajan, V.S., CRC. Crit Rev. Food Sci., 12,1979,199-301.
51. Ammon, H.P.T., and Wahl, M.A., Pharmacology of curuma longa,
Plant Med.,57,1991,1-7.
52. Srimal, R.C. and Dhawan, B.N., J. Pharmacol, 25,1973,447-452.

53. Stoskar, R.R., S.J., and Shenoy, S.G., *Int. J. Clin. Pharmacol. Ther. Toxicol.* 24,1986,651-654.
54. Sharma, O.P. *Biochem Pharmacol.*, 25,1976,1811-1812.
55. Toda, S., Miyase, T., Arichi, H., Tanizawa, H. and Takino, Y. *Chem Pharm. Bull. (Tokyo)*, 33,1985,1725-1728.
56. Nagabhushan, M., Amonker, A.J., Bhide, S.V., *Food Chem. Toxicol.*, 25,1997,545-547.
57. Nagabhushan, M., Bhide S.V., *J.Nutr. Growth. Cancer*, 4,1987,82-89.
58. Soudamini, K.K., and Kuttan, R.J. *Ethanopharmacol.*, 27,1989,227-233.
59. Azuine, M.A., and Bhide S.V. *Nutr. Cancer*, 17,1992,77-83,
60. Huang, M.T., Wang, Y.Z., Georgiadis, C.A., Laskin, J.D., and Connly, A.H. *Carcinogenesis*, 13,1992,2183-2186.
61. Huang, M.T., Smart, R.C., Wong, G.Q., and Cormey, A.H. *Cancer Res.*, 48,1988,5941-5946.
62. Deshpande, S.S., Ingle, A.D., Maru, G.B., *Cancer Lett.* 118,1997,79-85.
63. Deshpande, S.S., Ingle, A.D., & Maru, G.B. *Cancer Lett.*, 123,1998,35-40.
64. Bhide, S.V., Azuine, M.A., Lahirin, M. and Telong, N.T. *Breast Cancer Res. Treat.*, 30,1994,233-242.
65. Singletary, K., Macdonald, C., Wallig, M. and Fischer, C. *Cancer Lett.*, 103,1996,137-141.
66. Azuine, M.A., Kayal, J.J. and Bhide, S.V.J. *Cancer Res. Clin. Oncol.* 118,1992,447-45.

67. Singh, A.K., Sidhu, G.S., Deepa, T. and Mahaswari, R.K., *Cancer Lett.* 107,1996,109-115.
68. John, S.J., *AIDS Treatment News*, Vol. III, Gilden, D. Protease Inhibitor Overview and analysis, GMHC treatment issue, March 1994.
69. Kumar, S., Dubey, K.K., Tripathi, S., Fujii, M., and Misra, K., *Nucleic Acid Res. Sym. Ser.* 44,2000,75-76.
70. Coppock, D.L., and Paradee, A.B., *Mol. Cell Biol.* 198,1997,2925.
71. Reddy, G.P.V., and Paradee, A.B., *Nature*, 304,1983,86-88.
72. Madri, J.A. Bell, L., Marx, M., Mervin, J.R., Basson, C. and Prinz, C.J. *Cell. Biochem.* 45,1991,123-130.
73. Folkman, J., *J. Nutt Cancer, Inst.*, 82,1990,4-6.
74. Ross, R. and Harker, L. *Science*, 193,1976,1044-1049.
75. Pober, J.S., and Cotran, R.S., *Transplantation*, 50,1990,537-544.
76. Matsubara, T and Ziff, M.J. *Invert.* 79,1987,1440-1446.
77. Matsubara, T.Saura, R., Hiruhota, K. and Ziff, M. *J. Clin. Invert.*, 83,1989,158-167.
78. Harata, S., Matsubara, T., Saura, R., Tateishi, H. and Hirota, K., *Arthritis Rhum*, 32,1989,1065-1072.

CHAPTER 2

ANTIBACTERIAL ACTIVITY OF SOME

CURCUMIN BIOCONJUGATES

INTRODUCTION

Hospitalization predisposes the infected host to a wide variety of opportunistic pathogens. Common bacterial and fungal infections are frequent in cancer patients and immuno-compromised host. The significant morbidity and mortality resulting from these secondary infections are however largely overlooked. The emergence of wide spread distribution of drug resistant microorganisms have imposed serious limitations on successful antibiotic therapy. It is therefore required to explore alternative strategies for successful eradication of nosocomial infections.

Turmeric, an ancient spice belonging to the ginger family, is recently gaining enormous attention for its positive impact on a number of ailments. Curcumin, the major phenolic constituent of turmeric has been reported to have antioxidant properties^{1,2}. Turmeric and specially its major component curcumin are reported to inhibit carcinogen induced mutation in Ames assay³⁻⁷. These also inhibit the formation of tumor in several experimental systems suggesting anti-initiation and / anti promoting activity against carcinogens. Curcumin inhibits the proliferation of HUVEC (Human Umbilical Vein Endothelial Cells), therefore it could prove useful for the development of anticancer therapy⁸. It has also been reported that curcumin inhibits HIV-1 integrase protein, thus potentially preventing HIV-1 from infecting CD⁴ and CD⁸ cells⁹. Various potential applications associated with curcumin like anti-inflammatory property and their possible use against congenital and chronic viral diseases like cancer and AIDS have attracted considerable interest

during the past decade.^{10,11} The most important attractive feature of curcumin to be explored as vulnerary agent is that despite of being eaten daily for centuries in Asian countries curcumin has not been reported to be toxic.¹² Curcumin even though has positive impact on the prevention of such diseases, it cannot be used effectively due to its poor absorption through the intestinal wall and consequently required in large repetitive doses.

The objective of the present work was to evaluate *in vitro* antibacterial property of curcumin, turmeric peptide and some synthetic curcumin bioconjugates against gram positive and gram negative bacteria. Ten different bacterial strains viz *Staphylococcus aureus* , *Staphylococcus saprophyticus* , *Staphylococcus epidermidis* , *Streptococcus pyogenes* , *Micrococci* , *Peptococcus* , *Enterobacter cloace* , *Pseudomonas aeruginosa*, *E.coli* , *Klebsiella aerogenes* were selected on the basis of their resistance exhibited during β - lactam therapy for nosocomial infections and were responsible for numerous therapeutic failures and relapse of infection. It has been found that curcumin bioconjugates indeed show better activity against gram positive cocci as compared to curcumin itself. Good correlation was exhibited between MIC values and zone of inhibition .

MATERIALS AND METHOD

All solvents used were dried and distilled prior to use. Water was double distilled and autoclaved. Culture media were purchased from Hi Media Laboratories, Mumbai. Curcumin was purchased from Merck-Schuchardt Company, Germany. Turmeric peptide was isolated from aqueous extract of

Muller-Hinton agar (M1084) and broth (M391) were used for testing aerobic and facultative anaerobic bacterial isolates. For fastidious organism such as *Streptococci* and *Peptococcus*, the agar was supplemented with 5 % sterile, defibrinated blood.

Drug dilution: The solution of compounds were prepared in ethanol at an initial concentration of 100 g / ml and serially diluted to make an effective concentration of 25 mg / ml, 12.5 mg / ml, 6.25 mg / ml, 3.17 mg / ml, 1.58 mg / ml. Sterile disks (HI – Media) with 6 mm diameter were loaded with above dilutions. When dried, the discs were stored at 4°C.

Inoculation preparation :

3-10 colonies of the organism to be tested were picked with a wire loop from the stock and introduced into a test tube containing 4 ml of trypticase soya broth. These tubes were then incubated for 2 to 5 hr, to produce a bacterial suspension is diluted, with or saline solution to a density visually equivalent to that of a standard prepared by adding 0.5 ml of 1% BaCl₂ to 99.5 ml of 1% H₂SO₄ (0.36 N). Petri dishes were used with Muller – Hinton agar (5 to 6 mm in depth). Plates were dried about 30 min before inoculation.

MIC Determination: A standard platinum loopful (~ 0.001 ml) of the inoculum suspension was inserted deep into each tube of medium containing a known concentration of drug as well as drug free controls by a central down up motion. The tubes were tightly capped. A loopful of inoculum suspension

$$\frac{3774-20}{5895}$$

T-1017

was streaked onto Muller - Hinton agar to check the purity and viability. All cultures were incubated at 37⁰ C for 17 hrs.

End point criteria: The MIC's was defined as a point at which the organism was inhibited 80% compared with the growth in the control tube. All the isolates were seen in triplicate and the results were read visually. MIC's reported represent the results of at least two replications.

Disk diffusion method: The bacterial broth suspension was streaked evenly in three planes onto the surface of the medium with a cotton swab . Surplus suspension was removed from the swab by being rotated against the side of the tube before the plates are seeded . After the inoculum was dried (3 to 5 min) , the disk were placed on the agar with flamed forceps and gently pressed down to ensure contact . Plates were incubated immediately . After overnight incubation , the zone diameters (including the 6 mm disk) were measured with a ruler on the under surface of the Petri dish . A reading of 6mm indicates no zone ¹³⁻¹⁶.

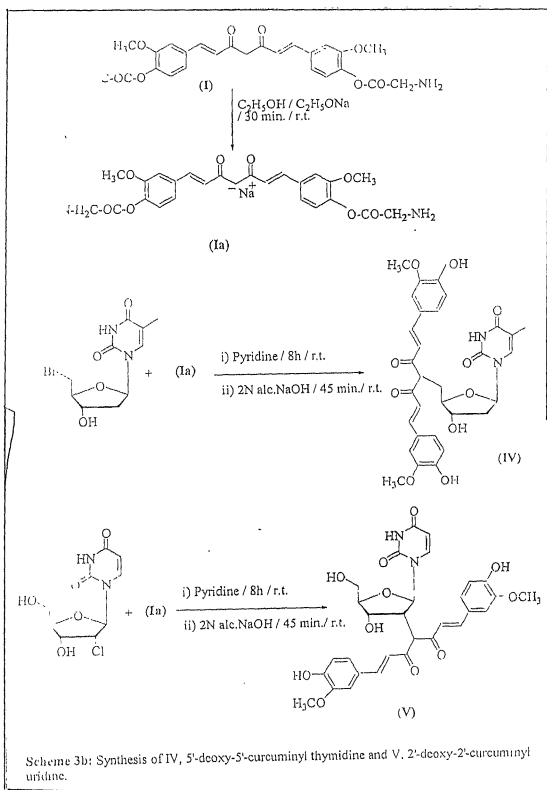
RESULTS:

Since curcumin shows extremely poor uptake, synthesis and characterisation of curcumin bioconjugates (I) - (IV) was undertaken mainly with nucleosides and amino acids. Curcuminbioconjugates (I) - (IV) were prepared in our laboratory (fig I). The rationale behind this was that the molecules linked to curcumin are known to pass easily through cell wall and hence when linked covalently to curcumin act as carrier molecules thereby increasing the intracellular concentration of curcumin.

In our preliminary *in vitro* investigations, number of gram positive and gram negative bacteria were used. Each test was performed in triplicate and MIC's

reported herein are results of at least two replications. It was found that out of four bioconjugates, three of them (I), (II) and (IV) show zone of inhibition of varying size against these multi-drug resistant microorganism. The most encouraging result was however obtained against *Streptococcus pyogenes* with (I) with MIC of 1.87 μ g / ml. This was compared with known antibiotic Amoxycylav which shows MIC of only 7 μ g / ml . Thus (I) has MIC three times higher than Amoxycylav. The turmeric peptide is resistant and show positive response only against an anaerobe viz *Peptococcus* having MIC 3.75 μ g/ ml. Another important result was found when MIC of synthetic bioconjugates were compared with that of curcumin and turmeric peptide. It was found that curcumin and turmeric peptide are resistant in most of the cases except against *Streptococcus pyogenes* where curcumin showed MIC 25 / ml. Thus in any case synthetic curcumin-bioconjugates (I) & (II) show greater MIC vis-à-vis curcumin (Table I).

Another significant result was observed when 30 μ g / ml of Amoxycylav and (I) were charged on separate disk followed by disk susceptibility testing. The zone of inhibition of (I) was found to be 28 mm which was considerably higher than that of Amoxycylav showing zone of inhibition only 20 mm (Table II)



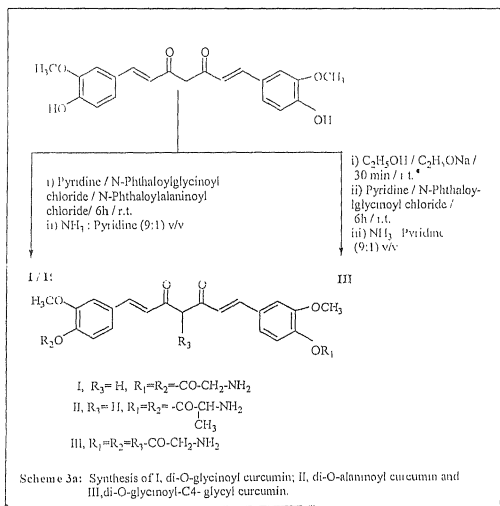


TABLE 1. MIC VALUES OF CURCUMIN AND ITS BIOCONJUGATES

S No	Name of Bacteria	I	II	IV	V	VI
1	<i>Enterobacter cloacae</i>	3.75	6.25	12.5	R	R
2	<i>Peptococcus</i>	3.75	6.25	12.5	3.75	R
3	<i>Staphylococcus epidermidis</i>	12.5	12.5	6.25	R	R
4	<i>Staphylococcus saprophyticus</i>	3.75	25	12.5	R	R
5	<i>Micro cocci</i>	3.75	12.5	3.75	R	R
6	<i>Staphylococcus aureus</i>	12.5	25	6.25	R	R
7	<i>Streptococcus pyogenes</i>	1.87	3.75	12.5	R	12.5

(I) Di-O-glycinoyl curcumin , (II), Di-O-glycinoyl-C⁴-glycyl-curcumin , (IV) 2'-deoxy-2'-curcuminyr uridine (V), Turmeric Peptide (VI), Curcumin,R Resistant (below 10 mm). Results with (III) 5'-deoxy-5'-curcuminyr thymidine (5'-cur-T) are not included,

MIC Values of Curcumin and its Bioconjugates

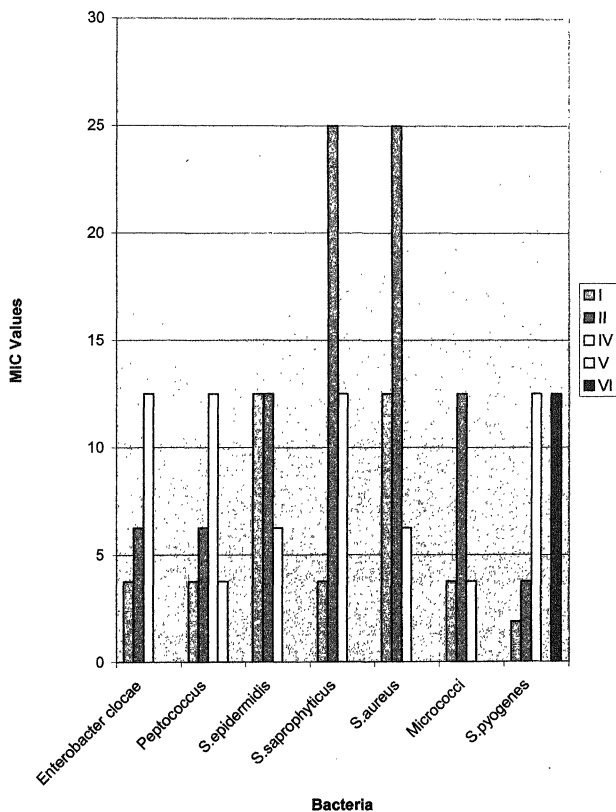
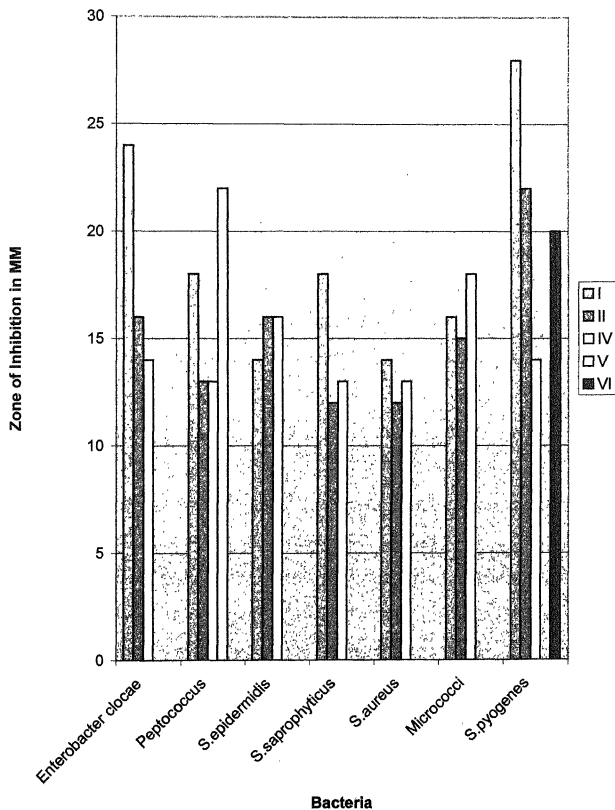


TABLE 2. Result of Zone of Inhibition in mm

S No	Name of Bacteria	I	II	IV	V	VI
1	<i>E. cloacae</i>	24,22,20,16	20,18,16	14,12	R	R
2	<i>Peptococcus anaerobe</i>	18,16 14,12	16,12, 11	13	22, 20 18, 12	R
3	<i>Staphylococcus epidermidis</i>	14,12	15,13	16,15, 14	R	R
4	<i>Staphylococcus saprophyticus</i>	18,16, 14,12	12	13,12	R	R
5	<i>Micro cocci</i>	16,15, 13,11	15,12	18,16, 14,12	R	R
6	<i>Staphylococcus aureus</i>	14,12	12	13,12, 11	R	R
7	<i>Streptococcus pyogenes</i>	28,24,20,18,16	22,24, 18,16	14,12	R	20, 16

(I) di-o-glycinoyl curcumin, (II) di-o-glycinoyl-C⁴-glycyl-curcumin, (IV) 2'-deoxy-2'-curcuminy l uridine,(V) Turmeric ,(VI) Curcumin, R Resistant (below 7mm).). Results with (III) 5'-deoxy-5'-curcuminy l thymidine (5'-cur-T) are not included.

Results of Zone of Inhibition



Discussion

To make drug delivery needle free researches have found ways to bypass drug absorption barriers in the skin and mucosa of intestines. Key advances have been made in chemical formulations not only to make drugs user friendly but to deliver higher concentrations of drugs at specific desired sites (targeted delivery). In order to achieve a certain effective concentration of drug within the targeted cell, there are ways for enhancing cellular uptake is its covalent linking with a molecule that is taken up routinely & can therefore drag the drug molecule along. However, the nature of the linkage must be such so that it is sensitive to the enzymes present leading to its cleavage & release of drug molecule at the desired site.

The micro-organism may be intrinsically resistant because of structural differences in the enzymes that are the targets of these drugs. One instance of bacterial resistance to the β lactam antibiotics are caused due to the former acquiring an enzyme (β -lactamase) that can cleave the β lactum ring by the inability of the agent to penetrate to its site of action. The positive result of (I), and (II) is probably due to the fact that glycine and alanine are natural components of cell wall of *Staphylococcus* and other gram positive bacteria. The significant result of (IV) is due to the fact that uridine is essential component of bacterial genome and hence well recognised. Curcumin-bioconjugate (III) gave results far from satisfactory which is due to the fact that thymidine is not a natural component of bacterial genome hence unable to internalise the molecule with the cellular environment of bacterial cell wall. Results also reveal that amino acid bioconjugates have been found to be more active than nucleoside bioconjugates due to several factors, one of these

being that bacterial cell wall probably allows those components to pass which have molecular weight less than 600, the amino acid bioconjugates have molecular weights in the range of 400-450 whereas nucleoside bioconjugates have molecular weight more than 550. In addition to the bulk problem, solubility plays a very important role. Amino acids being more hydrophilic as compared to nucleosides are more helpful to cellular uptake.

Curcumin bio-conjugates can emerge as a supplementary treatment modality in the subjects suffering from nosocomial infections. Curcumin being a natural product, has lesser or no side effects and the combination of bio-conjugates with these molecules can definitely change the amount of dosage, thus bringing about an altogether difference in the therapy of opportunistic infections. The results of the present study can thus be utilized for initiating a novel treatment for the subjects and thus bringing in much relief for the same.

CONCLUSIONS

From the results given above, it can be concluded that curcumin-bioconjugates show enhanced *in vitro* susceptibility against multidrug resistant bacterial strains. Since curcumin and turmeric show resistance in most of the cases, the results can be interpreted only in terms of enhanced cellular uptake through bacterial cell wall. This authenticates the objective of present work where enhanced cellular uptake can be achieved by designing conjugates with the ligands which can internalise the molecule with the cellular environment. In addition to cellular uptake, the ultimate fate of the drug is also important. The conjugates which act as pro drugs must contain covalent linkages which are

labile to the enzymes present in the cellular environment . The enzymic degradation liberates the drug at the desired site. The enhanced cellular uptake results in accumulation of the drug in the cellular environment to an extent which is necessary for disruption and lysing of the bacterial cell.

REFERENCES

1. .Srimal, R.C.and Dhawan , B. N.J.Pharmacol,25,1973,447-52.
2. Satoskar,R.R.,Shah,S.J.and Shenoy, S.G.Int. J. Clin. Pharmacol. Ther. Toxicol.,24, 1986, 651-654
3. Nagabhushan,M.,Amonker, A.J.,and Bhide, S.V.*Food Chem. Toxicol.*25,1987,545-47.
4. Nagabhushan,M.,,and Bhide, S.V.J.Nutr.Growth Cancer,4,1987,82-89.
5. Saudamini,k.k. and Kuttan,R.J.Ethanopharmacol.27,1989,227-33.
6. Azuine,M.A.andBhideS. V. Nutr.Cancer. 17,1992,77-84.
7. Haung,M.T.,WangY.Z.,etalCarcinogenesis13,1992,2183-86.
8. Anup K. SinghGurumel S.Sidhu etal Cancer Letter.107,1996,109-115.
9. John.S.J.,AIOS Treatment News vol
10. Sharma,O.P.Biochem.Biopharmacol .25,1976,1811-12
11. Toda,S.,Miyase,T., Arichi,H.,Tanizawa,H.and Takino,Y.,Chem. *Pharm .Bull.*(Tokyo),33,1985,1725-28.
12. A.,onH.P.T. and Wahl, H.A.,Pharmacology of Curcuma longa. Plant Med.57,1991,1-7.
13. A.W. Baur,W.M.M.Kirby,Antibiotic susceptibility testing by a standardized single disk method . Reprinted from Technical bulletin of the Registry of Medical technologists Vol36,No3,1966.
14. C.N. Baker,S.A.Stocker,D.H.Culver and C Thornsberry.Comparison of the E test to agar dilution , broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria.Jr.of Clinical microbiology Mar.1991,Vol29,3,533-538.

15. J.L.Burns,L.Saiman.Comparison of agar diffusion methodologies for antimicrobial susceptibility testing of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients,Jr of clinical Microbiology,May2000,Vol38,5,1818-1822.
16. L Martinez –Martinez,M.C.Ortega. Comparison of E-test with broth microdilution and disk diffusion for susceptibility testing of Coryneform bacteria.Jr.of Clinical Microbiology,May1995,1318-1321

CHAPTER-3

A CASE STUDY OF 200 PATIENTS OF

ONYCHOMYCOSIS

INTRODUCTION

The nail unit serves many useful functions such as protecting the digit , enhancing fine touch , and increasing tactile sensitivity . It is essential for picking up small objects, or performing activities such as fastening buttons . The nail unit is also an excellent tool for scratching and can be used as both an offensive and defensive weapon. Lastly, the nail unit is almost universally considered an important cosmetic attribute .¹ Thus any condition that adversely affects the nail unit may have deleterious consequences , affecting not only a person's ability to perform the daily tasks of living , but potentially diminishing his or her quality of life .²

ANATOMY OF NAIL

The nail consists of four epidermal components: matrix , proximal nail fold , nail bed and hyponychium .

The matrix, located deep in the finger tip , is in close proximity to the bony phalanx . This structure's horny end product is the nail plate , which moves distally to the nail bed . Most of the matrix is not visible ; only its distal portion , the lunula , could be seen as a white crescent – shaped structure projecting under proximal nail fold . Normally the nail plate is transparent and , like hair , has a different chemical makeup than the stratum corneum .

The proximally nail fold is a modified extension of the epidermis of the dorsum of the finger , which forms a fold over the matrix ; its horny end product is the cuticle . Because of its anatomical position , the cuticle is

deposited , on the surface of the newly formed nail plate . It normally desquamates immediately after it is formed .

The nail bed is an epidermal structure , beginning at the distal margin of the lunula and terminating with the hyponychium . It normally produces very small amounts of horny cells . Norton have demonstrated that there is a distal motion to the epidermal cells of the nail bed . The rate of this movement as compared to that of the nail plate has yet been undetermined .

The hyponychium is the extension of the volar epidermis under the nail plate and ends adjacent to the nail bed . The normal epidermal differentiation of these two nail structures (nail bed and hyponychium) is different morphologically , but becomes similar whenever the nail bed is involved in an abnormal process .

DEFINITION:

Onychomycosis includes all infection of the nail caused by dermatophytes , nondermatophytes and yeasts .

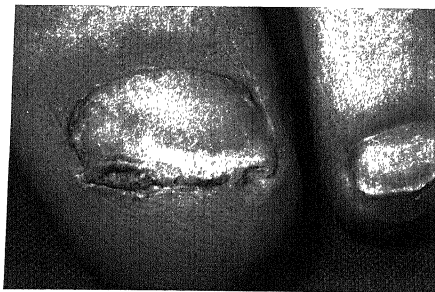
CLASSIFICATION:

Onychomycosis is generally classified according to the pattern of fungal invasion into the nail unit .4-6

- Distal Subungual Onychomycosis (D S O)
- White Superficial Onychomycosis (W S O)
- Proximal Subungual Onychomycosis (P S O)
- Candida Onychomycosis (C O)

DSO:

This type was first described by the younger Mahon ⁷ and is seen more commonly . In this type nail involvement occurs after invasion of the horny layer of the hyponychium and nail bed .



Pathodynamics:

The initial invasion was of stratum corneum , wheather from the hyponychium or the lateral nail fold , and subsequently of the nail bed . The nail plate appears normal during this early phase . The reaction of the hyponychium and the nail bed to the invading fungus is similar to that seen in epidermis elsewhere , i e , an acute or subacute dermatitis with increased thickening of the horny layers resulting in subungual keratosis . This horny thickening causes uplifting of the free edge of the nail plate with disruption of the normal

nail plate – nail bed attachment . Once this occurs , the presence of air under the nail plate produces the white appearance termed onycholysis . This is often seen in other nail conditions as well . An ecologic niche has been created , in which a varied micro flora coexists .

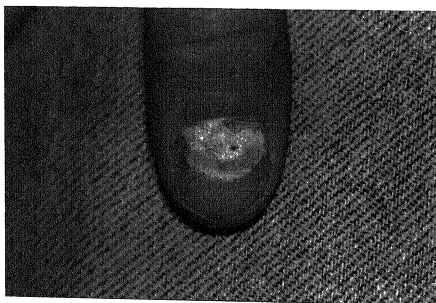
As the disease progresses , the offending organism advances proximally , growing on the stratum corneum of the nail bed and invading the undersurface of the nail plate . This may proceed rapidly or may take considerable time , depending on the pathogenic capabilities of the particular strain of the invading organisms . The excessive horn produced by the diseased nail bed epidermis can uplift the nail plate almost to a 45° angle . Subsequently , as the nail plate is more involved , it loses its innate transparency and the quality of its hardness may also change , becoming crumbly and dough like . Nail plate color has also been reported to change , appearing brown , green & gray . This however , is not the direct result of the primary invader , but rather due to coexisting microbial flora .

In the final stages of the process the abnormal nail plate either crumbles or is traumatically removed . Splinters of nail plate may remain over an exaggeratedly thickened nail bed . The diseased nail plate may continue to over grow the nail bed or the bed may remain without a nail plate for some time .

W S O :

W S O named leukonychia trichophytica by Jessner in 1922⁸ and leukonychia mycotica by Rost in 1926⁹ was first described in Germany and occurs as commonly as the D S O type . .

This clinical variety represents direct primary invasion and involvement of the nail by fungi . Clinically it appears as opaque , white , well demarcated islands on the surface of toe nail plates . These lesions , beginning as punctate forms randomly distributed , may coalesce and gradually involve the whole surface . Older lesions may have a yellow color . The surface of the toe nail is rough and its consistency is softer than normal nail plate , crumbling easily .



Pathodynamics

The fungal invades the nail plate on its superficial surface , forming islets which always remain in the most superficial layers of the plate . Invasion is the primary intension , as there are no soft keratinous layers (i e , stratum corneum) present . The morphology of the fungus is unique in that it is typical of a saprophyte , rather than the usual “parasitic” hyphal seen in stratum

corneum . Modified hyphal elements assuming various shapes , termed “eroding fronds” or “perforating organs,” are seen . These morphologic characteristics are always associated with hard keratinous structures , ie , hair feather , hoof and horn .

PSO :

This least common clinical type has been mainly reported in the European literature . Its characteristic clinical appearance consists of white areas extending distally from under the proximal fold , but basically confined to the areas of lunula . It may be seen in one or more finger or toe nail



Pathodynamics

The point of entry is thought to be at the proximal nail fold . The lesion is not on the surface of the nail plate , but rather in the deeper portions . As the nail plate grows , the white foci are carried distally . Large areas , eventually including the entire plate , may occasionally be involved . The fungus does not seem to evoke a severe inflammatory reaction in the underlying dermis of the

nail bed and matrix . Diagnosis is frequently missed because of unusual presentation .

CO :

Involvement of the nail plate by *Candida albicans* is rare . Diseased nails are opaque , noncrumbly , and often have longitudinal white strands with in the nail plate . Nail-bed thickening occurs and the distal digit appears bulbous or “pseudocluded .”



Pathodynamics

Invasion by *C. albicans* pseudohyphae throughout the entire length and thickness of the nail plate¹⁰⁻¹¹ in adjacent cuticle an hyponychial stratum corneum , and into the upper most layers of the stratum spinosum and in the granular layer in the abnormal nail bed epidermis . All the nail constituent are invaded by *C. albicans* , resulting in an abnormally thickened nail plate , massive inflammatory reaction in the proximal nail fold and nail bed ,and hyponychium inflammation which may account for the increase in fibrovascular tissue present producing a bulbous finger tip .

PREDISPOSING FACTORS:

A number of factors have contributed to the growing incidence of fungal nail infection , including an aging population , an expanding numbers of immunocompromised patients , increasing use of immunosuppressive drugs / therapies , use of occlusive footwear and communal bathing and changing room facilities for recreational purposes .There is also growing evidence that the pathogens involved in onychomycosis may be more diverse than previously believed .

THE NAIL PLATE AS SUBSTRATE

To a fungus the nail plate would seem to be very inhospitable habitat , being dry , indigestible and lacking in nutrients (Marples , 1965). The outer surface and distal end have , however one advantage ; they are far removed from the body's defence mechanisms . Other parts are closely integrated with , or spring from , the nail bed and nail fold and are therefore in closer contact with living cells and more likely to be affected by their activities . But these parts will have a higher moisture content , more nutrients , and consists of softer and therefore more easily digested keratin than the outer surface and nail tip . Fungi colonizes nails , therefore , must be able to adapt either to fairly close contact with the living host , or to extremes of desiccation and diet . The differing climates in which finger and toe nails provide another variable , at least in shoe wearing civilization ; that of toe-nails , kept warm and moist in encasing foot wear , would be less rigorous than that of finger nails . And pre existing disease or trauma may profoundly affect the chemistry of the keratin and therefore the ease with which it may be broken down . On the interplay of all these factors will depend which fungus is able to colonize any particular

nail or part of a nail and the form in which it will grow there , for different species have different climatic and nutritional requirements and are affected differently by proximity to the living body tissues .

ETIOLOGICAL AGENTS

Three groups of fungi are involved in onychomycosis : *the dermatophytes , the moulds and the yeasts* . Each group has characteristic growth requirements and limiting factors which differ from those of other groups ; with in each group some individual fungus species may have more or less specific requirements than others . It is to some extent possible to explain the clinical behaviour of fungi causing onychomycosis by relating these requirements and limitations to the varying habitats offered by different nails and different parts of the same nail .

IMMUNE MECHANISM AGAINST FUNGI

The potential allergenic implications of pathogen rich onychomycosis are currently under investigation. Onychomycosis is predominantly caused by *T. rubrum* , and onychomycotic nail harbor tremendous numbers of fungi that are compressed under the overlying nail plate . Cell Mediated Immunity may be suppressed in chronic *T. rubrum* infection by the mannan cell wall components . Despite being antigenic, it has been shown to inhibit lymphoproliferation stimulated by other antigens, such as *C. albicans* , tetanus toxoid , tetins ,and the anti-T-cell receptor antibody , anti-CD3 . Trichophytin can elicit a specific lymphoproliferative response, although it yields predominantly T-suppressor cells. Although cell mediated immune reactions may be suppressed by *T. rubrum* , the humoral response generally remains intact and antibodies of different classes have repeatedly been

demonstrated . Recently , Trichophyton –specific immunoglobulin E (IgE) antibodies were found in patients with T.rubrum infections . This may be significant in the atopic population . A causal relationship between onychomycosis , Trichophyton allergy , and asthma has been suggested and the potential hazard of nail dust inhalation by atopic pediatricians was also recently considered . It has been reported that atopic hand foot dermatitis in a patient who had immediate type hypersensitivity and specific IgE antibodies to trichophytin resolved only after successful systemic antifungal treatment of associated onychomycosis.

GENETICS

Because most patients with an onychomycosis have an affected parent . Zaia believed that the propensity to harbour dermatophytes and develop onychomycosis might be inherited as an autosomal dominant trait . Although cross –infection could also explain such familial aggregation , transmissibility of organisms does not seem to play a major part in determining the prevalence of Tinea pedis and D S O as studies amongst married couples where one partner is affected show little evidence of transmission to the unaffected partner.

Rippon postulated that chronic infection with T rubrum might also be determined by a genetic marker . Although many people are exposed to sources of T rubrum , and may experience an acute infection . Only a few selected T rubrum people become the constant reservoir of the organism for the community and also the vectors for its transmission to other areas .

Some dermatophyte infections such as tinea imbricata caused by *T concentricum* are associated with a genetic predisposition inherited in an autosomal recessive manner .

SPECIAL PATIENT POPULATION:

Onychomycosis in HIV positive patients

Onychomycosis in diabetic patients

Onychomycosis in the HIV – positive patients

Skin infections caused by dermatophytes are one of the most frequent dermatologic complications in patients with acquired immunodeficiency syndrome (AIDS) resulting from infection with human immunodeficiency virus (HIV) with an estimated prevalence of 15 – 40 %. There are even reports of immunocompromised patients who died of a disseminated fungal infection that began as onychomycosis caused by a nondermatophytes mold. The explanation of the increased incidence in this patient population is unclear . In these patients, toe nail, rather than finger nail involvement is more common. Furthermore, unlike other instances of onychomycosis, concurrent tinea pedis is uncommon. In one study, 55 of 61 HIV-infected patients with onychomycosis (88.7%) had proximal white subungual disease. *T. rubrum* was the cause in more than half of these patients. Although the actual incidence of tinea unguium in this population has not been determined it is accepted that the infection , although it can be an early manifestation of immunodepression , is more frequent when the CD 4 cell count approaches 450 cells / μ l and can present with a different clinical behavior than occurs with onychomycosis in

the general population . Thus , tinea unguium associated with AIDS is characterized by being clinically more aggressive , with rapid spread to the feet and hands having a higher frequency of unusual forms (PWSO) and possible periungual

ONYCHOMYCOSIS IN THE DIABETIC PATIENT:

Onychomycosis poses the greatest problem in the person with type I or type II diabetes who has sensory neuropathy and impaired circulation of the lower extremity. Onychomycosis results in thick, yellow brittle nails that can be sharp and pointed causing injury to the surrounding skin. The diabetic individual with neuropathy does not notice small cuts and breaks in the skin which can become a portal of entry for microorganisms. When ignored, these minor infections can escalate into serious paronychia and cellulites. Just as tight shoes can cause a friction blister in the neuropathic diabetic patient, thickened, dystrophic mycotic nails can cause pressure erosions of the nail bed and hyponychium. These skins break, unnoticed by the patient because of the sensory neuropathy , can result in serious limb-threatening infections. Several investigators have sought to determine whether the prevalence of onychomycosis is higher in diabetic patients. The consensus is that there is probably not an increased incidence of dermatophyte infections of the nail unit in diabetics, but the *candida* infections of the nail and surrounding area may be more prevalent in diabetics. The important point is that while onychomycosis may not be more common , the potential for serious sequelae is probably somewhat greater in diabetics than in nondiabetics.

MATERIAL AND METHODS:

200 clinically suspected cases of onychomycosis received from the skin clinic of Allahabad. In all the cases data related to the age , sex, duration of the lesions occupation , personal habits , family history of fungal infections were obtained . After a detailed clinical examination , the physical features of the nails were recorded . Care was particularly taken to record the presence of superficial mycotic infections on other parts of the body. Before obtaining a specimen , nails were cleansed by swabbing them liberally with alcohol to eliminate as many bacteria as possible, because they can overgrow and inhibit the growth of dermatophytes. Scrapings, clippings were collected from the deepest part of the nail(junction of the healthy and diseased portion of the nail). When both toe and finger nails were affected , specimen were collected from both the sites .In a patients with a presumptive diagnosis of distal subungual onychomycosis , a nail clipper was used to cut away the nail plate; then a curette was used to scrape the debris from the nail bed at a site as proximal to the cuticle as possible. A No 15 blade scalpel was used to scrape debris from the nail surface in the cases of WSO . In the cases of PSO healthy nail was pared back with a no 15 blade scalpel and a curette was used to remove the material from the proximal nail bed.

Each specimen was divided into two parts, one was taken for direct microscopic examination after 10 % KOH solution treatment & second was inoculated on sabouraud Dextrose agar (M286) & Sabouraud Cycloheximide Chloramphenicol Agar (M664) to facilitate the growth of all species. Two successive nail cultures were performed to establish the colonization of the pathogen because successive sampling rarely demonstrates the same contaminant.

Cultures were routinely incubated at 25-30 and examined daily for upto 4 weeks. The identification of individual fungi was based on standard methods such as

microscopic morphology, colonial characterization, pigment production, rate of growth & Biochemical tests.

RESULTS: 200 suspected cases of onychomycosis were investigated. Males outnumbered the females 131. The commonest age group affected was 21-30 years (Table 1). The duration of the lesions varied from 8 months to 5 years but in majority of cases it was less than one year. Out of 200 cases studied, the finger nails were affected in 20 cases. Toe nails in 35 cases and both toe and finger nails in 54 cases. The predominant clinical abnormalities observed were discolouration of the nail plate, subungual hyperkeratosis, leuconychia, total nail dystrophy, paronychia and pitting in that order of frequency (Table 2).

The common clinical types of nail involvement recorded were distal subungual onychomycosis in 98 cases, proximal subungual leuconychia in 38 cases, white superficial onychomycosis in 04 case and paronychia with nail lesion in 38 cases. (Table 3).

Mycological:

178 isolates were obtained on culture from the nails in the 200 patients giving a positivity rate of 89%. The predominant organisms identified were listed in (Table 4).

DISCUSSION

The maximum incidence of the disease was noted in the 21-30 years age group and in nearly all the age groups the males outnumbered the females. This

pattern of distribution has been noted commonly by many workers and could be due to more varied environmental exposures of males . The relative rarity of this condition in children was attributed to their faster linear nail growth and consequent inability of the fungus to get a proper hold in the nail substance . The most common nail abnormalities observed in this study were subungual hyperkeratosis and discolouration of the nail plate. The distal proximal subungual onychomycosis were the most common clinical types observed in this study and it is important to note that the white superficial type was seen only in one case. Among all the clinical types, candidal onychomycosis alone showed a predominance in females. Further, onycholysis was seen in 50% of the females whose occupation is exclusively domestic work. It is likely that domestic work is associated with constant trauma to the nails which hasten the destruction of an already diseased nail, resulting in onychomycosis.

The high recovery rate in this study could be due to the following reasons:

The scrapings were subjected to culture twice .This has led to better isolation of the pathogen.The cultural isolates revealed a predominance of *T. rubrum* over all other species. This may be explained on the basis of a rising trend in the incidence of *T. rubrum* infections with a decline in other species, particularly after the world wars, throughout the globe. Further, *T. rubrum* is unique among others in its special affinity for hard keratin such as the nails. *T. mentagrophytes* was the second common isolate (20%). Similar findings have been observed by many others also¹⁸ although a predominance of *T. mentagrophytes* was reported from the nails by a few workers. *T. tonsurans*

was isolated from two cases in the present study. In four cases Epidermophyton species were isolated.

Among the moulds, the Aspergillus species was isolated in 17 cases. In two of these cases, it was grown from the great toe nails of female patients who were accustomed to fashionable highheeled footwear. According to Rosman, such factors could lead to a constant pressure on the great toe nails decreasing their normal resistance to the invasion by moulds. In the third case, the mould was isolated from the finger nails of a patient who had culture positive dermatophytic skin infection. It is likely that the dermatophyte reduced the resistance of the finger nail keratin, paving the way for a mould. Among the yeasts, C. albicans was isolated in all the 7 cases seen with paronychia and nail changes.

It may be concluded that when many nails are involved, in association with mycotic skin lesions, the likely etiologic agent is a dermatophyte, commonly T. rubrum. In cases, where only a few nails are involved, or nails are involved in the absence of mycotic skin lesions, it is reasonable to suspect a mould. When the nail lesions are seen in the presence of paronychia, the causative agent is likely to be candida albicans.

Prevalence of onychomycosis in the diabetic population

Gupta and colleagues investigated the prevalence of onychomycosis in 550 patients with diabetes. They found clinically abnormal nails in 46% of patients and mycologic evidence of dermatophyte infection in 26% of patients. The

presence of onychomycosis was correlated with age and with mail gender . In fact , males with diabetes were 2.99 times more likely to have onychomycosis than females with diabetes. The overall risk ratio of individuals with diabetes having onychomycosis is 2.77 compared with age and sex- matched nondiabetic controls.In the present study out of 6 diabetic patients 4 were suffering from onychomycosis.

Prevalence of onychomycosis in HIVdisease

According to the recent study from Ohio , which is representative of industrialized nations where occlusive footwear is commonly worn, the prevalence of onychomycosis is 13.7%. In the 1996-1997 study from Strasborg, the prevalence of onychomycosis was similar (12.6%) in controls but was 30.3%in individuals infected with HIV.In the present study 2 patients were presented the picture of onychomycosis .



TABLE –I Age and sex distribution in 200 cases of Onychomycosis

Age in Years	Males	Females	Total
1-10	2	3	5
11-20	5	16	21
21-30	25	17	42
31-40	4	33	37
41-50	5	18	23
51-60	12	15	27
61-70	6	12	18
71-80	-	5	5

TABLE II CULTURE ISOLATES IN RELATION TO THE SITE OF INVOLVEMENT

FUNGUS ISOLATED	FINGER NAIL	TOE NAIL	BOTH	SKIN
Dermophyte				
<i>T.rubrum</i>	35	05	12	8
<i>T.mentagrophyte</i>	20	08	10	-
<i>T.tonsuran</i>	04	01	03	-
<i>T.violacium</i>	02	01	-	01
<i>Epidermatophyton</i>	04	-	-	-
Non Dermophyte				
<i>Acremonium</i>	13	-	-	-
<i>Fusarium</i>	07	-	-	-
<i>Scopulariopsis</i>	08	-	-	-
<i>Aspergillus terreus</i>	15	-	-	-
<i>Aspergillus fumigatus</i>	02	-	-	-
<i>Absidia</i>		-	-	-
<i>Rhizopus</i>	01	-	-	-
Yeast				
<i>C.albicans</i>	07	-	-	-
<i>C.para</i>	01	-	-	-
<i>C.tropicalis</i>	02	-	-	-
<i>Rhodotorula</i>	04	-	-	-

TABLE 3Clinical type and culture isolates in 200 cases of
onychomycosis

CLINICAL TYPE	TOTAL	D	ND	Y	M
DSO	98	20	40	8	30
PSO	10	2	8	2	10
WSO	4	-	4	-	-
CO	38	-	-	15	23

REFERENCES:

1. Scher.R.K.Onychomycosis:A significant medical disorderJr. Of the American Academy of Dermatol1996;35(suppl 2)
2. Scher.R.K.Onychomycosis is more than a cosmetic problem.Br J Dermatol 1994;130(suppl 43):15
3. Zaias N.Onychomycosis .Arch Dermatol 1972 ;105,263-274
4. Norton L.A.:Incorporation of thymidine-methyl-H3 and glycine-2-h3in the matrix and bed of the human.J Invest Derm1971:56,61-68.
5. Mahon, quoted by Rosenthal T:Early nineteenth century dermatology and the brothers Mahon. Arch Derm Syph1950:30,245-250
6. Jessner.M:Leuchonychiatrachophytica.ArchDerSyph.(Berlin)1922:141, 1-8
7. Roast G. R.Hauthrankheiten.Verlag1926:74
8. Rippon.J.W.Elastase : Production by ringworm fungi.Science.1967:157:947.
9. Zias N. superficial white onychomycosis.Sabouradia :1966 :5,99-103.
10. English M.P.Saprophytic growth of keratinophilic fungi on keratin .Sabouradia 1963:115-130.
11. Stuhmer A: Subungual Epidermatophyton Trichophytic and favus. Arch Derm Syph 1952:193,527-536.
12. Grimmer H; Histopathologie and therapie.1960:28,365.
13. English M.P.Nails and Fungi. British J of Derm .1976,94,697.
14. Marples,Mary J.(1965) The ecology of the human skin.

CHAPTER-4

TWENTY NAIL DYSTROPHY: A CASE STUDY

OF 50 PATIENTS

INTRODUCTION

The term trachonychia was coined by Alkiewicz in 1950 to record cases of rough nails.¹ Achten & Wanet-Ronard (1974) took up the term in a classification of nail dystrophies of known and unknown cause².

Hazelrigg, Duncan & Jarrat (1977) described a group of children in which excess ridging was the principle feature and named the condition 'the twenty nail dystrophy of childhood'³. Following this article Baran & Dupre (1977) in a letter to the Archives of Dermatology pointed out that some cases are probably due to alopecia areata but where the whole nail is not involved Lichen planus or psoriasis may be responsible ⁴. Peter(1979) classified nail dystrophy in three groups:

1. Alopecia areata or totalis
2. Twenty nail dystrophy of child hood
3. Severe nail dystrophy

Clinically, however, there is very little difference between the nail changes in the three groups but group 2&3 were considered idiopathic.⁵ Twenty nail dystrophy is a distinctive pattern of nail changes , in which all nails are uniformly affected .Because of the rarity of this disorder, and controversy surroundings its cause and pathogenesis , we share our experience with fifty patients whose clinical diagnoses were consistent with twenty nail dystrophy .

MATERIAL AND METHODS:

50 clinically suspected cases of twenty nail dystrophy attending a skin clinic of Allahabad were studied. In all the cases data related to the age, sex, duration of the lesions, occupation, personal habits, family history of fungal infections were obtained. After a detailed clinical examination, the physical features of the nails were recorded. Care was particularly taken to record the presence of superficial mycotic infections on other parts of the body. Before obtaining a specimen, nails were cleansed by swabbing them liberally with alcohol to eliminate as many bacteria as possible, because they can overgrow and inhibit the growth of fungi. Scrapings, clippings were collected from the deepest part of the nail (junction of the healthy and diseased portion of the nail). Specimen from both toe and finger nails were collected, a nail clipper was used to cut away the nail plate; then a curette was used to scrape the debris from the nail bed at a site as proximal to the cuticle as possible. A No 15 blade scalpel was used to scrape debris from the nail surface.

Each specimen was divided into two parts, one was taken for direct microscopic examination after 10 % KOH solution treatment & second was inoculated on Sabouraud Dextrose agar (M286) & Sabouraud Cycloheximide Chloramphenicol Agar (M664) to facilitate the growth of all species. Two successive nail cultures were performed to establish the colonization of the pathogen because successive sampling rarely demonstrates the same contaminant.

Cultures were routinely incubated at 25⁰–30⁰C and examined daily for up to 4 weeks. The identification of individual fungi was based on standard methods

such as microscopic morphology, colonial characterization, pigment production, rate of growth & Biochemical tests.^{6,7}

RESULTS:

In the present study 50 cases of twenty nail dystrophy were evaluated . Males outnumbered the females.Age and sex distribution is mentioned in the table no 1.Out of fifty cases only thirty three cases were positive for fungus. Rest seventeen cases were sterile after one month incubation . The various fungi isolated are mentioned in table no 2 Out of thirty three culture positive cases, the most common pathogen isolated was *Candida albicans* followed by non dermatophytes and dermatophytes.

STATISTICAL ANALYSIS :

The above following data may be tabulated in the following table:

Frequencies			
Observed values (O)	Expected values (E)	$(O-E)^2$	$(O-E)^2 / E$
Positive-33 Cases	$50 * 2/3 = 33.33$	0.109	0.0033
Negative-17 Cases	$50 * 1/3 = 16.66$	0.09	0.0054
$\Sigma r = 50$			$\Sigma (O-E)^2 / E$ = 0.0087

The value of chi square will be 0.0087 at 1 d.f. 5% From the above data the tabulated value of chi-square at 1 d.f. 5% is 3.841 which is much higher than calculated value at 1 d.f. 5% is 0.0087 So the data gave the good fit to the ratio of 2:1 and calculated values are significant i.e., observed values are agreed with expected values.

LE -1 AGE AND SEX DISTRIBUTION IN TWENTY NAIL DYSTROPHY

AGE	FEMALE	MALE	TOTAL
0-10	01	03	04
11-20	00	04	04
21-30	01	04	05
31-40	03	08	11
41-50	00	06	06
51-60	01	02	04
TOTAL	06	27	33

Statistical analysis

Segregation data of male and female ratio in 50 cases of TND female (12)

0.0814 and male (21) 0.0407 value of chi-square is 0.122

RESULTS:

The calculated value of chi -square for idf 5% is 0.122 (probably) which is less than from tabulated value at idf 5% 3.841. Therefore results are significant i.e. observed value is agree with expected value the ratio 1:2 is true.

TABLE-2 ISOLATED PATHOGENS

FUNGUS	NAIL
<i>T.rubrum</i>	02
<i>T.mentagrophyte</i>	04
<i>T.tonsuran</i>	01
<i>Scopulariopsis brevicaulis</i>	02
<i>Aspergillus terreus</i>	03
<i>Fusarium</i>	01
<i>Acremonium</i>	02
<i>Absidia</i>	01
<i>Rhodotorula</i>	01
<i>Candida albicans</i>	09
<i>Candida tropicalis</i>	02
<i>Candida albicans T.mentagrophyte</i>	03
<i>Candida albicans Acremonium</i>	01
<i>Candida albicans Candida para</i>	01

DISCUSSION:

In the present study, cases under consideration showed no evidence of other skin disorders like psoriasis, lichen planus and alopecia areata. The condition had remained unchanged and had been unresponsive to a wide range of therapies .Almost half of the cases showed positive cultures of Candida its species and other moulds which strongly indicates their likely role in the pathogenesis of 20 nail dystrophy. Though our work is still preliminary nevertheless significance of this work can not be overlooked .

REFERENCES:

1. Achten, G. & Wanet-Rouard, J. (1974) Archives Belges Dermatologie, 30, 201.
2. Alkiewicz, J. (1950) Trachyonychia. Annales de dermatologie et de syphiligraphie, 10, 136.
3. Baran, R. & Dupre, A. (1977) Vertical striped sand paper nails (letter). Archives of Dermatology 113, 1613.
4. Hazelrigg, D.E., Duncan et al. Twenty nail dystrophy of childhood. Archives of dermatology 113, 73.
5. Wilkinson, J.D. et al (1979) Twenty nail dystrophy of childhood. Case report and histopathological findings. British Jr. of dermatology, 100, 217.
6. Gerald E. Pierard, Jorge E. Arrese. Present and potential diagnostic techniques in onychomycosis. Jr. Am Acad Dermatol. 1996; 34: 273-7
7. Boni E. Elewski. Diagnostic techniques for confirming onychomycosis. Jr. Am Acad Dermatol. 1996; 35: S6-S9.

CHAPTER-5

ANTIMYCOTIC ACTIVITY OF SOME

CURCUMIN BIOCONJUGATES

INTRODUCTION

Onychomycosis is a common fungal infection of one and more components of nail unit and accounts for about fifty percent of the nail associated disorders¹⁻³. The toenail infection is more prevalent as compared to fingernail infection and it may affect as many as 48% of the population by age 70 and can be quite severe in some patients, particularly in those with AIDS and the elderly⁴⁻⁶. In India the prevalence of onychomycosis is equally alarming and many investigations have been carried out in this field. The infection is due to increased exposure to the infecting organisms and also due to greater use of immunosuppressive and anti-biotic agents, acquired immunodeficiency syndrome and increased incidence of diabetes mellitus⁷. The infection may be associated with significant pain and discomfort and in severe cases may result into disfigurement and loss of dexterity and mobility. Besides clinical implication it may also impose certain psychological / social limitations⁸. The fungal infections of the nail are notoriously difficult to treat, spontaneous remission is rare and recurrence after treatment has traditionally been common.⁹

The causative agents are to a large extent dermatophytes such as *T. rubrum*, *T. mentagrophytes*, followed by yeasts mainly *Candida albicans* and non-dermatophytes viz. *Scopulariopsis*, *Scytalidium*, *Acremonium*, *Aspergillus* and *Fusarium*^{1,2,7}. The treatment of onychomycosis is difficult because of unique absorption properties of nail unit. To be effective, drug must penetrate the affected tissue and remain in high concentration till the pathogen is eradicated. Topical anti-fungal drugs have restricted use due to poor

absorption. Systemic treatment with griseofulvin, ketoconazole, itraconazole and fluconazole gives unsatisfactory results, as these are associated with narrow spectrum activity, require prolonged treatment and have low cure rates⁸. Recent understanding of the pharmacokinetics associated with these drugs has led to the use of several successful drugs like Fluconazole, Itraconazole ,terbinafine etc. Nevertheless, need to explore alternative strategies for the treatment of onychomycosis still exists.

Curcumin has been used conventionally for variety of medicinal applications and is known to have antioxidant, anti-inflammatory, anti-cancer, antibacterial and antiviral properties¹⁰⁻²¹. However effective use of curcumin is limited due to the facts that it shows very poor absorption through intestinal membrane and hence is required in large and repetitive doses²². There are various ways to enhance the cellular uptake of drug molecules, the most important being their covalent linking to such biomolecules which being inherent to the system can carry the drug along the cell. Once inside the cell the enzyme present can hydrolyze the biodegradable linkage, releasing the free drug at the desired site. This is presently targeted drug delivery via bioconjugates. Therefore curcumin could be modified by covalent attachment with certain ligand to promote its entry into the pathogen. Curcumin-bioconjugates prepared by covalently linking curcumin to molecules like glycine or uridine did show enhanced cellular uptake. The most probable explanation is that the ligands attached to curcumin are such through the cell wall of the microorganism and thus can internalise the conjugate with the cell environment, resulting in its drag through the cell wall. The covalent linkage used in these bioconjugates is the ester linkage which is likely to cleave in the

presence of esterases, present in the cytoplasm thus releasing the drug at the required site (targeted delivery). Therefore, these bioconjugates can be called as prodrugs. These bioconjugates can serve dual purpose of systemic delivery as well as therapeutic activity against viral/bacterial/fungal diseases. In our laboratory some bioconjugates have been designed, prepared and characterised viz. di-O-glycinoyl curcumin (I), di-O-glycinoyl-C-4-glycyl-curcumin (II), 5'-deoxy-5'-curcuminyl thymidine (III) and 2'-deoxy-2'-curcuminyl uridine (IV). These, along with sodium salt of curcumin (V), turmeric peptide (VI), turmeric (VII) and curcumin (VIII), have been evaluated for their antifungal activity against in vivo fluconazole resistant onychomycotic agents. This indicates that curcumin bioconjugates show enhanced cellular uptake through fungal cell wall at comparatively lower concentration vis-à-vis curcumin.

Literature survey however suggests that neither curcumin nor any of its bioconjugates have so far been evaluated for their antifungal properties. In the present work (I), (V), (VI), (VII) and (VIII) have been evaluated for their antifungal properties. The fungi used are causative agents for onychomycosis and includes *Trichophyton rubrum*, (dermatophytes), *Acremonium*, *Aspergillus* (non dermatophytes) and *Candida albicans* (yeast). The objectives of this study were (i) to determine the MIC's; (ii) to perform the zone of inhibition and (iii) to correlate the results of MIC's and zone of inhibition. Thus, the overall objective was to assess the antifungal property of these curcumin bioconjugates against those organisms which have developed resistance against Fluconazole, the well known antifungal agent.

MATERIAL AND METHODS

Isolates:

FR strains were obtained from the cases of onychomycosis received from the skin clinic of Allahabad. The nail clippings and scrapings were cultured on sabourad dextrose agar and sabourad dextrose broth and were maintained on slants at -70°C until they were tested, identification of fungi was based on established standard methods.

Medium:

Sabourad dextrose agar and sabourad dextrose broth were used for disc diffusion method and macrodilution method.

Drug dilution:

The solution of compounds were prepared in ethanol at an initial concentration of 100mg/ml and serially diluted to make an effective concentration of 25mg/ml , 12.5mg/ml , 6.25mg/ml .

Sterile disk (HI — Media) with 6 mm diameter were loaded with above dilutions. When dried, the discs were stored at 4°C .

Inoculum's Preparation:

A suspension that was just turbid (0.5 McFarland standard) by visual inspection was prepared by suspending the selected yeast in sterile water. Mycelia growth of filamentous fungi was preferred when heavy particles persisted after vortexing and they were allowed to settle and homogenous suspension was used for inoculation.¹⁸

MIC Determination:

A standard platinum loopful (0.001 ml) of the inoculum suspension was inserted deep into each tube of medium containing a known concentration of drug as well as drug free control by a central down up motion. For filamentous fungi sterile mineral oil (0.5 ml) was layered on the inoculated medium to inhibit sporulation. The tubes were tightly capped. A loopful of inoculum suspension was streaked onto sabourad dextrose agar to check the purity and viability. All cultures were incubated at 30⁰ C until good growth was apparent from second day to seventh day.¹⁹

End point criteria:

The MIC's was defined as a point at which the organism was inhibited 80% compared with the growth in the control tube. All the isolates were seen in triplicate and the results were read visually. MIC's reported represent the results of at least two replications.

Disk Diffusion:

Sabourad dextrose agar was poured to a depth of 5mm in 150 mm petri dishes and stored at 4⁰ C. The plates were dried, the standardised inoculum (0.001 mm) suspension poured and uniformly spread. Plates were allowed to dry for 5 minutes. The disk were then applied and the plates were incubated at 30⁰ C for seven days. The diameter of the clear zones around the disks was measured with a scale. Two readings were taken at right angles and two disk on separate plates were used for each drug and averaged to determine the zone diameter for a given isolates.¹⁹

RESULTS:

Literature survey suggests neither curcumin nor of its bioconjugates have so far been evaluated for their antifungal activity . We have tested these bioconjugates in vitro against the onychomycotic agents viz *Trichophyton rubrum* , *Acremonium* , *Aspergillus*, *Candida albicans* which were resistant to Fluconazole , a standard drug for onychomycosis .The results shown in table I are quite encouraging . The work done on Curcumin, it's four bioconjugates and it's Sodium salt in a two year study showed that bio-conjugates no. 1 and Sodium salt of curcumin has a wider range of zone of inhibition in comparison to curcumin while the other three bio-conjugates II , III & IV were found to be resistant against the fungal isolates.(As mentioned in Table No.1) Likewise the MIC values of the bio-conjugate I and Sodium salt of curcumin is lower in comparison to that of Curcumin. Since the other bio-conjugate II , III & IV were seen to be resistant hence the MIC value of the three bioconjugates could not be evaluated (As seen in Table No.2). The above results thus, make a strong correlation between the MIC value and the zone of inhibition. This ultimately brings about a difference in the amount of dosage of curcumin bio-conjugates in the treatment of Onychomycosis.

TABLE-I RESULTS OF ANTI FUNGAL ACTIVITY OF CURCUMIN BIOCONJUGATES BY DISC DIFFUSION METHOD (ZONE OF INHIBITION IN MM)

FUNGUS	I	II	III	IV	V	VI	VII
<i>Trichophyton rubrum</i>	15	R	R	R	R	10	10
<i>Acromonium</i>	12	R	R	R	R	12	8
<i>Aspergillus terreus</i>	R	R	R	R	R	R	R
<i>Candida albicans</i>	12	R	R	R	R	7	12

(I) Di-O-glycinoyl curcumin , (II), Di-O-glycinoyl-C⁴-glycyl-curcumin , (III) 5'-Deoxy-5'-curcuminyl thymidine , (IV) 2'-deoxy-2'-curcuminyl uridine ,(V) Turmeric Peptide (VI), Curcumin , R- Resistant (below 7 mm),(VII) Sodium salt of curcumin.

Results of Zone of Inhibition

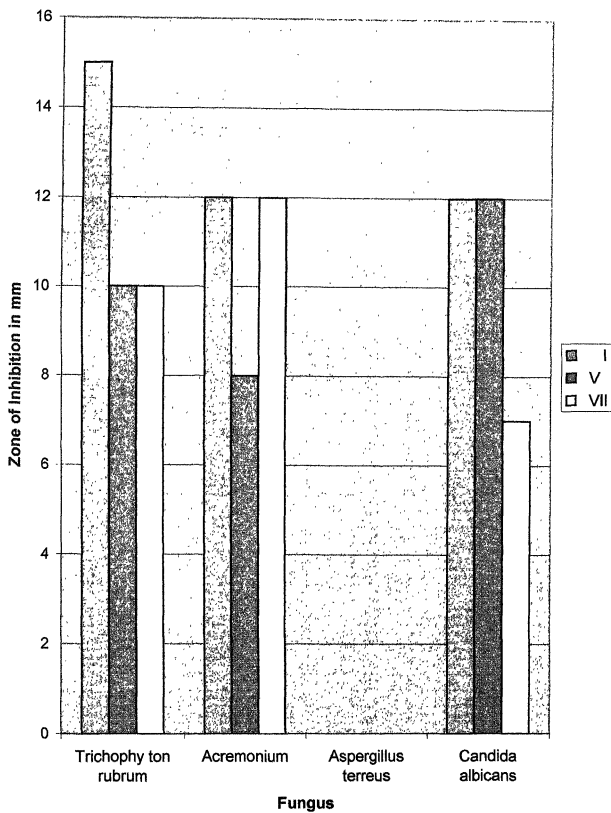
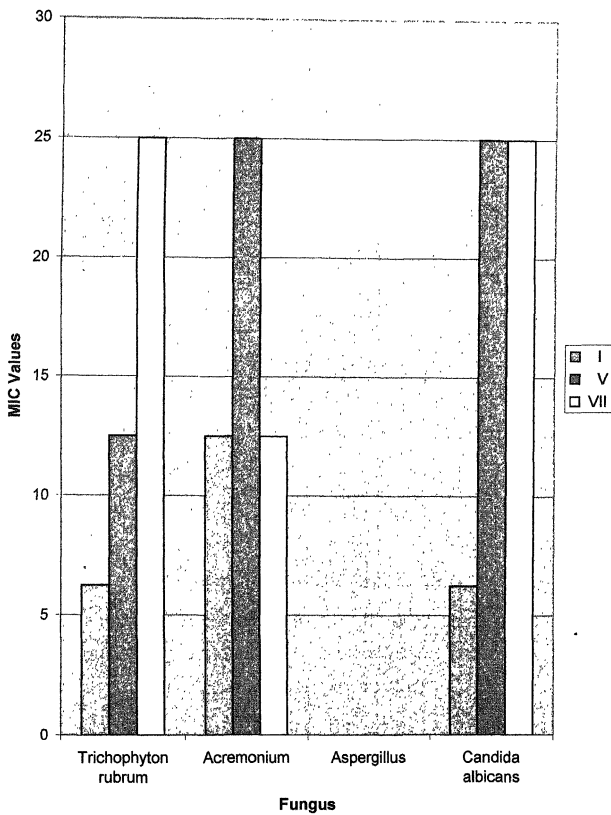


TABLE II MIC VALUES OF CURCUMIN BIOCONJUGATES

FUNGUS	I	II	III	IV	V	VI	VII
<i>Trichophyton rubrum</i>	6.25	R	R	R	R	25	12.5
<i>Acromonium</i>	12.5	R	R	R	R	12.5	25
<i>Aspergillus</i>	R	R	R	R	R	R	R
<i>Candida albicans</i>	6.25	R	R	R	R	25	25

(I) Di-O-glycinoyl curcumin , (II), Di-O-glycinoyl-C⁴-glycyl-curcumin , (III) 5'-Deoxy-5'-curcuminy l thymidine , (IV) 2'-deoxy-2'-curcuminy l uridine, (V)Turmeric Peptide ,(VI)Curcumin, R- Resistant (below 7 mm),(VII)Sodium salt of curcumin

MIC Values of Curcumin Bioconjugates



DISCUSSION

Curcumin albeit is known to have variety of clinical applications but has not proved effective enough mainly because of its poor absorption through the intestinal wall. To make it effective at lower concentration, it is necessary to enhance the uptake of curcumin through the biological membranes. We made an effort to increase its cellular uptake by binding it covalently with a moiety which being essential component of the pathogen's cellular environment could pull it inside the cell, thus causing significant increase in the intracellular concentration. This approach for improving the uptake of the curcumin molecule involves a design that could allow the enzyme-mediated transformation of the bioconjugates within the target organ. These conjugates particularly one with amino acids is possibly sensitive to some intracellular enzymes and hence once inside the cell can release the drug molecule at the desired site. In our earlier work, synthesis and characterisation of curcumin bioconjugates (I) - (IV) was carried out. These were evaluated for their *in vitro* susceptibility against β -lactamase producing strains of bacteria (published work). It was found that curcumin-bioconjugates indeed show better antibacterial activity against gram positive cocci vis-à-vis curcumin. This authenticates the idea that conjugates of curcumin with amino acids and nucleosides are better internalised with the cellular system and hence show enhanced cellular uptake as compared to curcumin itself. The hydrophilic nature of these bioconjugates may also help in active transport across cellular membrane.

Nail Clipping's from suspected cases of onychomycosis referred from a skin clinic in Allahabad were cultured to investigate the effect of curcumin-

bioconjugate (I)-(IV) developed by us against the causative fungi and compared with (V) - (VIII) (Fig 1). The most prevalent causative organisms viz. *T. rubrum*, (dermatophytes), *Acremonium*, *Aspergillus* (non - dematophytes) and *Candida albicans* (yeast) were selected. It can be observed that (I) showed greater zone of inhibition as compared to the rest. It can also be observed that sodium salt of curcumin (V) showed positive response but in poor range as compared to (I). While (VI), (VII) and (VIII) were resistant.

MICs evaluated for (I) in all the four cases were found 6.25 , 12.5 , R, 6.25 μ g/ml. which is much better than MICs of Curcumin 25 , 12.5 , R , 25 μ g/ml under similar conditions It can therefore be concluded that curcumin-bioconjugate (I) shows better absorption through fungal cell wall and hence show better results even at lower concentration.

From our results it can be concluded that curcumin-bioconjugates show better anti-fungal activity vis-a-vis curcumin which is possibly due to enhanced cellular uptake through fungal cell wall. Though our work is still in its infancy and includes limited number of causative organisms nevertheless significance of this work cannot be ignored. It opens a new avenue for exploring suitably designed curcumin bioconjugate as potential antifungal agents. More such bioconjugates can be designed by keeping one of the components common to either the fungal cell wall or with fungal genome conjugated with curcumin , thereby improving the efficiency of curcumin at comprehensively low doses. These may prove to be better systemic drugs by concentrating mainly on and around the malignant cells by recognizing the cell wall of fungus .

The common antibiotics available in market today for fungal infections may develop resistance quickly and possibly become ineffective. Therefore, need to develop inexpensive drugs with negligible toxicity is quite pressing.

REFERENCES

1. Ramesh V, Reddy BSN, Singh R. Onychomycosis. *Int J Dermatol* 1983 ;22:148-152.
2. Zias N. Onychomycosis. *Arch Dermatol* 1972;105:264-274.
3. Summerbell RC. Epidemiology and ecology of onychomycosis. *Dermatol* 1997;194:32-36.
4. Elewki B, Charif LM. The prevalence of onychomycosis inpatients attending a dermatology clinic in north eastern Ohio for other conditions. *Arch Dermatol* 1997;133:1172-3.
5. Zaias N. Clinical manifestation of Onychomycosis. *Clin Exp Dermatol* 1992 ; 17 (suppl 1) : 6-9 .
6. Domp Martin D, Domp Martin A et al Onychomycosis and AIDS : clinical and laboratory findings in 62 patients. *Int Jr of Dermatol* 1990; 29:337-9.
7. Zaias N, Glick B, Rebell G . Diagnosing and treating onychomycosis. *J Fram Pract* 1996 ;42:513-8.
8. 10.Srimal, R.C.and Dhawan , B. *N.J.Pharmacol*,25,1973,447-52.
9. Satoskar,R.R.,Shah,S.J.and Shenoy, S.G.*Int. J. Clin. Pharmacol. Ther. Toxicol.*,24, 1986, 651-654
10. Nagabhushan,M.,Amonker, A.J.,and Bhide, S.V.*Food Chem. Toxicol.*25,1987,545-47.
11. Nagabhushan,M.,,and Bhide, S.V.*J.Nutr.Growth Cancer*,4,1987,82-89.
12. Saudamini,k.k. and Kuttan,R.*J.Ethanopharmacol.*27,1989,227-33.

13. Azuine, M. A. and Bhide S. V. *Nutr. Cancer*. 17, 1992, 77-84.
14. Haung, M. T., Wang Y. Z., et al *Carcinogenesis* 13, 1992, 2183-86.
15. Anup K. Singh Gurumel S. Sidhu et al *Cancer Letter*. 107, 1996, 109-115.
16. John. S. J., *AJOS Treatment News* vol
17. Sharma, O. P. *Biochem. Biopharmacol* .25, 1976, 1811-12
18. Toda, S., Miyase, T., Arichi, H., Tanizawa, H. and Takino, Y., *Chem. Pharm .Bull.* (Tokyo), 33, 1985, 1725-28.
19. A., on H. P. T. and Wahl, H. A., *Pharmacology of Curcuma longa. Plant Med.* 57, 1991, 1-7.

LIST OF PUBLICATIONS :

PUBLISHED

1. Synthesis of curcumin – bioconjugates and study of their antibacterial activity against Beta lactamase producing Micro-organisms. Sanjay Kumar, Upma Narain , Snehlata Tripathi and Krishna Misra .Bioconjugate Chemistry.

COMMUNICATED

1. Onychomycosis : Role of Nondermatophytes . Upma Narain , A.K. Bajaj , Krishna Misra . Jr. of the American Academy of Dermatology.
2. Twenty Nail Dystrophy : Role of Fungi casual & causal. Upma Narain , A.K. Bajaj , Krishna Misra .European Jr. of Dermatology.
3. Review: Onychomycosis . . Upma Narain , A.K Bajaj , Krishna Misra .Indian Jr. of Dermatology.
4. Comparative *in vitro* Property of Curcumin and Curcumin-Bioconjugates against Multi-drug Resistant Micro-organism . Upma Narain , Sanjay Kumar Krishna Misra .International Jr. of Antimicrobial Agents .
5. Preliminary Evaluation of *in vitro* antifungal activity of some curcumin-bioconjugates against *in vivo* fluconazole resistant onychomycotic agents Upma Narain , Sanjay Kumar Krishna Misra. International Jr .of Clinical Microbiology.

Paper presented in symposia/conferences/workshop:

1. Sanjay Kumar, Upma Narain and Krishna Misra .Bioconjugate Synthesis of curcumin – bioconjugates for targeted drug delivery.Indo-russian Seminar,23-26 Jan. 2000,Delhi University ,Delhi.
2. Sanjay Kumar, Upma Narain and Krishna Misra. curcumin – bioconjugatesas potential bactericides,AIBA Biotech Symposium26-27Feb. 2000,Hyderabad.